

**An Investigation into the Direct  
and Indirect Effects Induced by  
Platelet-Activating Factor Receptor  
Activation in Rat Isolated Lung Tissue.**

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## **Abstract**

This study looked at the effects of platelet-activating factor (PAF) on rat isolated lung strips and tracheal spirals. Previous studies have shown that PAF induces hyperresponsiveness to the contractile effects of acetylcholine (ACh) and 5-hydroxytryptamine (5-HT) in rat isolated tracheal spirals. In this study it was hoped that previous experiments on tracheal spirals could be repeated and then be extended to investigate similar responses in lung tissue. It would then be possible to make a direct comparison between the effects of PAF on the larger airways with the effects of PAF on the smaller airways.

It has also been observed in previous studies that PAF induces a desensitization of its receptors to the bronchoconstrictor effects of PAF. This study looked at whether this PAF receptor desensitization occurred in rat isolated airway tissue. This was done by adding PAF to tissue preparations and observing its effects on the tissues, then adding a subsequent dose of PAF to see if responses were repeated. With the use of inhibitors of protein kinase C (sphingosine), phospholipase A<sub>2</sub> (AACOCF<sub>3</sub>) and lipoxygenase (MK886) which have all been implicated as mediating the actions of PAF, it was possible to determine possible mechanisms responsible for the desensitization of PAF receptors. This was done by introducing these inhibitors into the experimental protocol before addition of PAF and then removing them before subsequent addition of PAF. One theory behind PAF receptor desensitization is receptor internalisation, and this was investigated with the use of an immunofluorescence (IF) technique. This IF technique was then

developed to investigate the possibility of eosinophils entering lung tissue as a result of the method of killing the animal.

In this study hyperresponsiveness of tracheal and lung tissue to the contractile effects of ACh and 5-HT in response to PAF receptor activation was not observed. Desensitization of tissues to the bronchoconstrictor effects of PAF was however observed. The use of IF technique indicated that in the case of rat isolated airway tissue, receptor internalisation was not responsible for PAF receptor desensitization. The use of MK886 indicated that desensitization was also not the result of leukotriene production, whilst the use of sphingosine indicated that protein kinase C activation was not responsible for desensitization. The results suggested that desensitization of tissues to the effects of PAF after an initial PAF application was the result of arachidonic acid production or, more likely, either down-regulation of receptors or receptor/G-protein/effector uncoupling after phospholipase A<sub>2</sub> activation.

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### **Declaration**

I confirm that this thesis is my own work and that sources of any information used (including the internet) have been fully identified and properly acknowledged.

Signed: \_\_\_\_\_

Stephen Peter Welsh

## **Chapter 1**

### **Introduction**

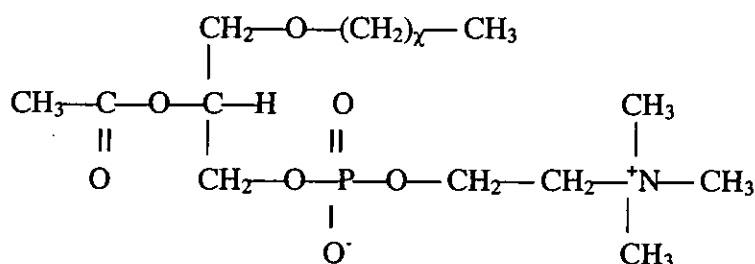
#### **1.1 General**

This study investigated the effects of platelet activating factor (PAF) on the airways. PAF is secreted from the airways in asthmatics (Chung and Barnes, 1991) and it has been suggested in previous work, that it causes bronchoconstriction and may make the airways hyperresponsive to neurotransmitters such as acetylcholine (ACh) and to 5-hydroxytryptamine (5-HT) (Warhurst & Court, 1996). As asthma is a disease characterised by airway inflammation, excessive airway secretions and bronchoconstriction, anything causing bronchoconstriction and making the airways more responsive to neurotransmitters, must be treated as a potential major contributor to the symptoms of the disease. It was for this reason that the contractile response of PAF *per se* was investigated along with the effects of PAF on the contractile response induced by ACh and 5-HT.

#### **1.2 History of PAF and Discovery of Structure**

In 1970, a soluble factor was released from leukocytes, which caused platelets to aggregate (Henson, 1970). This substance was found in another study (Benveniste *et al.*, 1972) and was shown to be a polar lipid. The substance was named platelet-activating factor (PAF). At around the same time, other research described an antihypertensive polar renal lipid (APRL) produced in the renal medulla (Muirhead *et al.*, 1977). By 1979, enough evidence had been accumulated to conclude that PAF and APRL were structurally identical (see figure 1); 1-*O*-alkyl-2-acetyl-*sn*-glycerol-3-phosphocholine

(Demopoulos *et al.*, 1979). Shortly afterwards another phospholipid, acetylglyceryletherphosphorylcholine (AGEPC) with the same chemical and biological properties as PAF was synthesized (Hanahan *et al.*, 1980). Subsequently, PAF, APRL and AGEPC were all found to be exactly the same (Polonsky *et al.*, 1980).



**Figure. 1 Platelet-activating factor.**

Where  $\chi = 11-17$

### 1.3 PAF Synthesis

There are two metabolic pathways involved in the biosynthesis of PAF, the remodelling and the *de novo* pathways (see figure 2). In the remodelling pathway, PAF is synthesized from its substrate in two steps (Chilton *et al.*, 1984). The first step involves the action of phospholipase A<sub>2</sub> (PLA<sub>2</sub>), with the formation of lyso-PAF and arachidonic acid (AA). In some cells (e.g. eosinophils) this reaction represents a major source of AA that is metabolized to thromboxanes, prostaglandins and leukotrienes. In the second step, lyso-PAF is acetylated by acetyl coenzyme A, in a reaction catalysed by lyso-PAF acetyltransferase, to form PAF. Both the phospholipase and acetyl transferase are dependent on Ca<sup>2+</sup> (Hanahan, 1986., and Snyder, 1989). The inactivation of PAF also occurs in two steps (Chilton *et al.*, 1983). Initially, the acetyl group of PAF is removed by PAF acetylhydrolase to form lyso-PAF which is present in both cells and plasma. This

lyso-PAF is then converted to a 1-*O*-alkyl-2-acyl-glycerophosphocholine by an acetyltransferase in a step inhibited by  $\text{Ca}^{2+}$ .

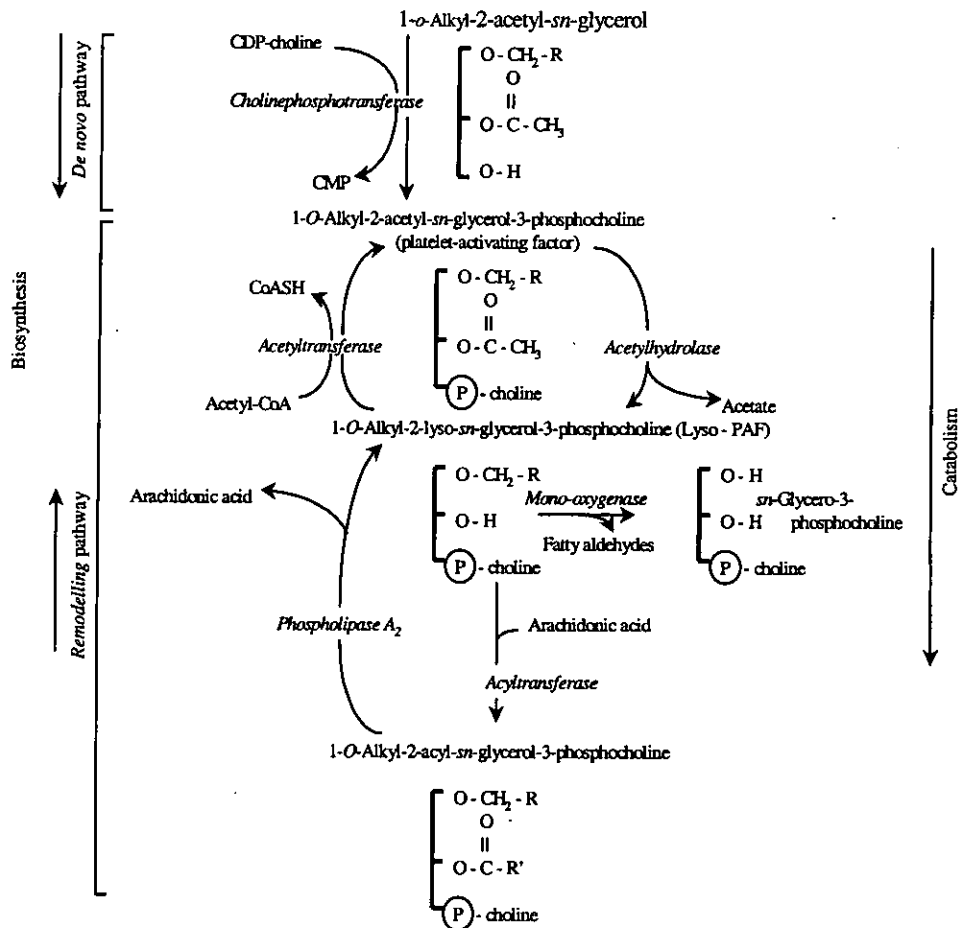


Figure 2. The *de novo* synthesis and remodelling metabolic pathways of PAF. (Chao and Olson, 1993)

R and R' = fatty acids (P) = Phosphate group

## 1.4 Cells Producing PAF

PAF is a phospholipid produced by alveolar macrophages, endothelial cells and granulocytes, especially blood basophils, after appropriate stimulation (Kingsnorth, 1996). PAF is an important proinflammatory mediator which causes microvascular

leakage, vasodilation, contraction of smooth muscle, activation of many cell types and plays an important role in allergic disorders, inflammation and endotoxic shock (Kingsnorth, 1996). PAF is released in acute asthma, and may be a mediator of antigen-induced airway obstruction (Henson & Pinckard, 1977).

### **1.5 PAF and Hyperresponsiveness of Smooth Muscle**

PAF has been shown to cause hyperresponsiveness of airway smooth muscle to methacholine in humans (Cuss *et al.*, 1986), hyperresponsiveness has been observed in guinea pig airways (Yukawa *et al.*, 1989) and an enhanced response to stimuli was observed in rabbit platelets pretreated with PAF (Henson, 1976). Kelly *et al.* (1989) found PAF in bronchoalveolar lavage (BAL) fluid from some asthmatics. This and the finding that stools from patients suffering from Crohns disease contained increased quantities of PAF (Denizot *et al.*, 1991) suggest that PAF has a role in human disease states. In each case it was suggested that the PAF was associated with the inflammation present. This study therefore used lung tissue for subsequent investigation.

### **1.6 PAF Induced Bronchoconstriction via Protein Kinase C**

Previous work (Berridge, 1987., and Nishizuka, 1986) has suggested that one way in which PAF may affect bronchoconstriction is via a G-protein leading to the activation of protein kinase C (PKC). Following activation of the PAF receptor, a number of processes are triggered via a G-protein linked cell surface receptor (Braquet *et al.*, 1987). These include; the activation of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) leading to arachidonic acid (AA) release (Chao and Olson, 1993); and the production of diacylglycerol (DAG) and inositol

1,4,5-tris-phosphate (IP<sub>3</sub>). IP<sub>3</sub> leads to an increase in cytosolic Ca<sup>2+</sup>, and DAG in the presence of this calcium activates protein kinase C (PKC) which leads to cell activation (Henderson, 1991).

### **1.7 PAF Induced Bronchoconstriction via Phospholipase A<sub>2</sub>**

Following PLA<sub>2</sub> activation, AA is released and is then broken down by either cyclooxygenase to form prostaglandins and thromboxanes, or by lipoxygenase, to form leukotrienes. Eicosanoids (breakdown products of AA) may therefore function as extracellular mediators of the effects of PAF. Thromboxane A<sub>2</sub> has been shown to be a potent vasoconstrictor, and has been shown to cause constriction in vascular smooth muscle (Bhagwat *et al*, 1985). Some prostaglandins (PGF's and PGD<sub>2</sub>) have also been shown to cause constriction in smooth muscle although other prostaglandins (PGE<sub>1</sub> and PGE<sub>2</sub>) have been shown to relax airway smooth muscle (Spannhake *et al*, 1981). Leukotrienes C<sub>4</sub> (LTC<sub>4</sub>), D<sub>4</sub> (LTD<sub>4</sub>) and E<sub>4</sub> (LTE<sub>4</sub>) are extremely potent bronchoconstrictors in many species including man (Piper, 1984). They act principally on smooth muscle in peripheral airways, and LTC<sub>4</sub> and LTD<sub>4</sub> are at least 1000 times more potent than histamine *in vitro* and *in vivo* (Dahlen *et al.*, 1980). In this study, the effects of leukotrienes as bronchoconstrictors were investigated as rat airways were used. Previous studies have shown PAF to have its contractile effect in the airways via leukotrienes in the rat (Voelkel *et al.*, 1982). Furthermore, the cysteinyl leukotrienes have already been implicated in the pathogenesis of asthma (Ind, 1996).

## 1.8 Formation of Leukotrienes

PAF stimulates phospholipase A<sub>2</sub> (PLA<sub>2</sub>) which causes the release of lyso-PAF which can be used in the generation of PAF. AA is commonly also generated as it is often the acyl grouping removed from the precursor during lyso-PAF generation. This AA can then be used to produce LT's. The enzyme 5-lipoxygenase uses AA and lyso-PAF to produce leukotrienes and bring about contraction as follows: 5-lipoxygenase in the presence of Ca<sup>2+</sup> is translocated from a site within the cell to the nuclear envelope where it is fully activated by 5-lipoxygenase-activating protein (FLAP) (Henderson, 1991). FLAP facilitates the transfer of AA from the membrane phospholipase to 5-lipoxygenase. The expression of 5-lipoxygenase and FLAP is required in cells to catalyze the oxygenation of arachidonic acid generating 5-hydroperoxyeicosatetraenoic acid (5-HPETE), the key intermediate in the formation of leukotrienes. After 5-HPETE proton abstraction and dehydration leukotriene A<sub>4</sub> (LTA<sub>4</sub>) is formed in a reaction, also catalyzed by 5-lipoxygenase (Samuelsson *et al.*, 1987). LTA<sub>4</sub>, AA, and glutathione (glutathione only used for LTC<sub>4</sub>) are then converted by LTA<sub>4</sub> hydroxylase or LTC<sub>4</sub> synthase, to produce either leukotriene B<sub>4</sub> (LTB<sub>4</sub>), or LTC<sub>4</sub>. LTC<sub>4</sub> is then converted by the actions of enzymes cleaving glutamic acid and glycine to form leukotrienes D<sub>4</sub> and E<sub>4</sub> (Henderson, 1991). In human isolated bronchus preparations, LTC<sub>4</sub> and LTD<sub>4</sub> have proven to be extremely potent at causing smooth muscle contraction (Dahlen, S-E *et al.*, 1980). There are two classes of receptor for leukotrienes, leukotriene B (BLT) receptors and cyteinyll (CysLT) receptors. There are two groups of CysLT receptors, which are CysLT<sub>1</sub> and CysLT<sub>2</sub> receptors. CysLT<sub>1</sub> receptors can be blocked by known antagonists whilst CysLT<sub>2</sub>

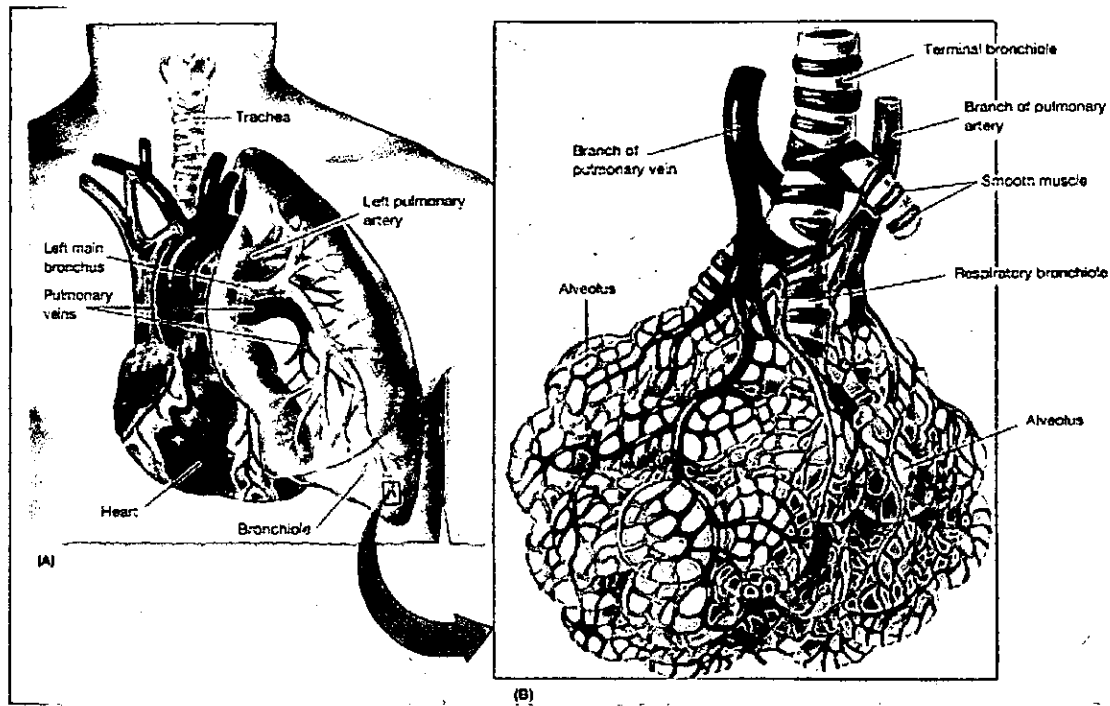


receptors cannot (Ind, 1996). In airway tissue, LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub> all activate CysLT<sub>1</sub> receptors, which in turn brings about bronchoconstriction.

## **1.9 PAF and Eosinophils**

Airway hyperresponsiveness in asthma has been linked to eosinophilic inflammation in the airways (Gleich *et al.* 1988), and the increased responsiveness seen after exposure to PAF may be a result of the ability of PAF to induce eosinophil infiltration and activation. Activation of eosinophils by PAF results in a rapid rise in intracellular calcium followed by the release of enzymes such as eosinophil peroxidase and cyclooxygenase (Kroegel *et al.*, 1988 and 1989). PAF also induces the formation of hypodense eosinophils, which are found in asthma, by degranulating them (Yukawa *et al.*, 1989). The mechanism by which PAF leads to degranulation appears to involve the entry of Ca<sup>2+</sup> via receptor-operated channels and stimulation of phosphoinositide hydrolysis to release Ca<sup>2+</sup> from intracellular stores (Yukawa *et al.*, 1989). Activated eosinophils appear to cause shedding of ciliated epithelium, as is seen in asthma, via the release of eosinophil peroxidase and basic proteins (Yukawa *et al.*, 1990). Epithelial damage may lead to bronchial hyperresponsiveness in a number of ways. These include the loss of an epithelial-derived relaxant factor, the activation of exposed airway sensory nerves by mediators, the production of inflammatory mediators or the loss of enzymes such as neutral endopeptidase which degrades bronchoconstrictor peptides (Cuss and Barnes, 1987).

## 1.10 Structure of the Airways



**Figure, 3. Organisation of the respiratory system. A) Indicates the positioning of the trachea and lungs in the body and gives some indication of the small size of the alveoli, and hence how many there are. B) Shows in detail the structure of the bronchioles and alveoli that make up the vast majority of the lungs. (Vander, *et al.*, 1994).**

The human respiratory system is made up of two lungs, each divided into several lobes and the airways that lead to them. The lungs consist mainly of millions of tiny air sacs called alveoli, through which gas exchange with the blood occurs. The airways are the tubes through which air flows between the alveoli and the external environment. Air entering the lungs passes first through the pharynx (throat), then through the larynx, trachea, bronchi (one of which enters each lung), bronchioles, terminal bronchioles, respiratory bronchioles, alveolar ducts and finally the alveolar sacs (see figure 3). The walls of the trachea and main bronchi contain supportive cartilage, giving them shape. The bronchioles begin where this cartilage stops (Horsfield, 1974).

### **1.11 Innervation of the Airways**

The walls of the airways contain smooth muscle cells, which are innervated by autonomic neurons. These autonomic neurons are further broken down into the sympathetic and parasympathetic divisions. The sympathetic nervous system brings about relaxation in the lungs, whilst the parasympathetic brings about contraction. Both systems stimulate airway secretion. The parasympathetic nervous system has nerve endings in the epithelium which respond to a variety of mechanical and chemical stimulus. These receptors are responsible for the reflex actions to inhaled irritants and to some respiratory disease processes such as asthma (Widdecombe, 1982). Stimulation of these receptors may cause changes in breathing including defensive reflexes, airway mucus secretion or changes in airway calibre.

### **1.12 Neurotransmitters in the Airways**

In both parasympathetic and sympathetic nervous systems, the major neurotransmitter between pre and post-ganglionic fibres is acetylcholine (ACh). The major neurotransmitter between the postganglionic fibres and effector cells in the parasympathetic nervous system is ACh, and in the sympathetic nervous system, noradrenaline (NA). The ACh receptors in all smooth muscle, such as that which makes up the respiratory system, are muscarinic receptors (van der Velden and Hulsmann, 1999). Smooth muscle is also sensitive to certain other neurotransmitters such as 5-hydroxytryptamine, (5-HT) (Burns and Doe, 1978).

### **1.12.1 Acetylcholine**

Acetylcholine (ACh) is made up from choline and acetyl coenzyme A in the cytoplasm of synaptic terminals and is stored in synaptic vesicles. From here it is released to activate receptors on the postsynaptic membrane which in turn activates effector cells and brings about muscle contraction. The concentration of ACh at the postsynaptic membrane is reduced (stopping receptor activation) by the enzyme acetylcholinesterase, releasing choline to be transported back into axon terminals for the production of new ACh (Andersson and Grundstrom, 1987). ACh will be used in this study as it has been shown to markedly contract rat isolated airways (Akcasu, 1952).

### **1.12.2 5-Hydroxytryptamine**

The biogenic amines are neurotransmitters synthesized from amino acids. The most common neurotransmitters of this type are dopamine, NA, histamine and 5-hydroxytryptamine (5-HT). 5-HT is produced from tryptophan, and is metabolised by monoamine oxidase. Its effects generally have a slow onset, suggesting that it may act as a neuromodulator (Burns and Doe, 1978). In general 5-HT has an excitatory effect on the efferent nerve pathways involved in smooth muscle contraction (Hahn, 1986). 5-HT will be used in this study as it has previously been shown to produce contractions in rat isolated airway preparations (Burns and Doe, 1978) whilst other neurotransmitters such as histamine have been found to be ineffective in rat isolated airway preparations (Akcasu, 1952).

### **1.13 Asthma**

Asthma is a common condition affecting approximately 5% of the adult population and over 10% of children in the western world. Asthma is an example of a chronic airway disease. It is a disorder in which airway inflammation, excessive airway secretions and bronchoconstriction increase airway resistance, impairing ventilation. It is airway inflammation which causes smooth muscle to become hyperresponsive, making it contract strongly in cases of, for example, exercise, stress, extreme temperatures or exposure to respiratory irritants (Lemanske and Busse, 1997). This additional resistance in the airways is overcome by increasing inspiratory effort with the use of muscular contraction, forcing gasses out of the lung. The result of the increased resistance is however, a decreased respiratory reserve, hence the oxygen content of alveolar gas and of the blood decreases, whilst carbon dioxide levels increase. This makes physical exertion more difficult, including the muscular contraction needed to increase inspiratory effort (Leff, 1982).

### **1.14 Mast Cells in Asthma**

Increased levels of mast cells in the lung in the late phase asthmatic response alongside increased levels of specific mast cell derived mediators in bronchoalveolar lavage fluid in patients with asthma (Bingham and Austen, 2000) implicate the mast cell in the pathogenesis of asthma. The mast cell may respond to activating stimuli through its receptors for immunoglobulins and various polypeptide ligands. In response to these activating stimuli, which may be derived from other cells in the environment, the mast cell exocytoses pre-formed mediators stored in secretory granules and synthesises

substantial quantities of newly formed lipid mediators including PAF, from membrane phospholipid-derived substrate (Bingham and Austen, 2000). This mast cell derived PAF along with other mediators, interacts with other cells such as eosinophils to increase vascular permeability, cause bronchoconstriction, increase airway mucus secretion and recruit other cells into the site to amplify the inflammatory response (Clifton *et al.*, 2000).

### **1.15 PAF in Asthma**

Early reports on the causes of asthma found a significant drop in the platelet count after an allergen provocation in sensitive patients (food, inhalents or drugs), which was transient (Pohle and Cohen, 1947). It was shown that this could in part be due to the release of platelet-activating factor (PAF) in the lung followed by a transient platelet pooling in the pulmonary circulation and the desensitization of platelets to PAF (Beer *et al.*, 1995). The early asthmatic response results in PAF release, and raised levels of leukotrienes are detected in tissues and fluids from asthmatic patients compared with normal individuals (Sampson *et al.*, 1992).

PAF is released in acute asthma and may be a mediator of antigen-induced airway obstruction (Henson and Pinckard, 1977). PAF is not stored in cells but is synthesised by inflammatory cells such as macrophages, eosinophils and platelets in response to stimulation (Pinckard *et al.*, 1982). Observations suggesting a role for PAF in asthma include; i) PAF is a potent activator of inflammatory cells including eosinophils (Kroegel *et al.*, 1989) and platelets (Kloprogge and Akkerman, 1984); ii) infusion of PAF to laboratory animals induces bronchoconstriction, which resembles the pathological

features of asthma (Maridonneau-Parini *et al.*, 1985); iii) inhalation of PAF by normal subjects as well as patients with mild asthma induces an immediate bronchoconstrictive response and may stimulate the airways to non-specific stimuli such as methacholine (Cuss *et al.*, 1986); and iv) a deficiency in PAF acetylhydrolase, which rapidly inactivates PAF in plasma, closely correlates with the severity of asthma in children (Miwa *et al.*, 1988).

## 1.16 Tissues Used

Using lung and tracheal tissue in this investigation will allow the results to be used as a closer comparison to the effects of PAF in asthmatic lung tissue, which may have some bearing on asthma. No attempt was made however to mimic an asthmatic state. Rat tissue was used in this investigation, the reason for which is that in for example the guinea pig, IgG is the primary antibody responsible for mediating anaphylactic bronchoconstriction. In the rat however, as in man, allergic bronchoconstriction is mediated primarily through antigen combining with IgE (Lulich and Paterson, 1980). As stated earlier, human lungs are each divided into several lobes, in rats the lungs are divided into four lobes, with the left lung being undivided.

Rat isolated lung and tracheal tissue was used because;

- a) it has been shown to express the PAF-receptor gene (Bito *et al.*, 1994);
- b) rat lungs have been shown to release lipid mediators which more closely resemble the release pattern observed from human lung than other animal models appear to release (Voelkel *et al.*, 1985);

- c) previous work undertaken in this Department using rat isolated lung tissue enables a comparison of the initial data and provides expertise in a number of the techniques which will be required to be used.

### **1.17 PAF and Desensitization of Smooth Muscle to PAF**

Previous work has also suggested that PAF causes various tissues to become desensitized to the effects of PAF after an initial application; guinea pig ileal smooth muscle (Findlay *et al.*, 1981), rat stomach fundus (Levy, 1987) and rat vascular smooth muscle cells (Schwertschlag & Whorton, 1988). Consequently this study was used to confirm the desensitizing action of PAF on airway smooth muscle and to investigate the mechanism through which PAF induced this selective desensitization.

Beer *et al.*, 1995, showed that PAF receptors become desensitized to the effects of PAF after an initial application as demonstrated by the desensitization of platelets in asthmatic patients (Beer *et al.*, 1995 and Chesney *et al.*, 1985). Desensitization has also been demonstrated in rabbit platelets (Henson, 1976), rat mesenteric arterial bed (Kamata *et al.*, 1996) and guinea pig lung (Honda *et al.*, 1991). A number of mechanisms have been suggested as being responsible for this desensitization (Kamata *et al.*, 1993).

This study will therefore look at whether desensitization to PAF after an initial exposure occurs in the airways, and if so what mechanisms may be involved. Ligand-induced desensitization can be divided into two categories: agonist-specific (homologous) desensitization or agonist non-specific (heterologous) desensitization. Homologous



desensitization is where only the subsequent response to the desensitising drug is affected, whilst the effects of other drugs are not affected. Heterologous desensitization is where the effects of the desensitising drug and other drugs are affected (Sibley *et al.*, 1987). PAF induced desensitization has previously been found to be of the homologous type (Kamata *et al.*, 1996), a finding which was confirmed in this study.

### **1.18 Desensitization as a Result of Protein Kinase C Activation**

As mentioned earlier, there are a number of theories for the mechanisms of PAF desensitization although which is correct is not currently understood. In a study into the effects of PAF on the contractile response in rat stomach fundus (Kamata *et al.*, 1993) it was suggested that activation of protein kinase C leads to receptor phosphorylation and desensitization. This is supported by other work which looked at the role of PKC and receptor function in rabbit iris smooth muscle (Abdel-Latif *et al.*, 1986). If this were the case, mechanisms resulting in desensitization could involve direct phosphorylation of receptors (Sibley *et al.*, 1987), inactivation of the receptor-phospholipase link (Smith *et al.*, 1987) or direct interference with  $\text{Ca}^{2+}$  channels (Galizzi *et al.*, 1987). Studies with rabbit platelets however, showed that protein kinase C is not involved in PAF desensitization (Shimizu *et al.*, 1992). They showed that responses produced by  $\text{IP}_3$  remained unchanged before and after PAF application. It was also shown by Nakamura *et al.*, 1991, that protein kinase C was not involved in the desensitization of the platelet-activating-factor receptor in rabbit platelets.

### **1.19 Desensitization as a Result of Receptor/G-Protein/Effector Uncoupling**

It was therefore suggested that desensitization could be the result of down-regulation of the receptor, or to the impairment of receptor/G-protein/effector coupling, but not of depletion of the  $\text{Ca}^{2+}$  store (Nakamura *et al.*, 1991). The theory of G-protein uncoupling being responsible for the desensitization of PAF receptors is supported by the work of Kamata *et al.*, (1996). G-proteins become dissociated from the PAF receptor when PAF binds to the receptor causing GTP to bind to an  $\alpha$ -subunit of the G-protein, activating the G-protein. The G-protein then dissociates from the receptor, causing the affinity of the receptor for the agonist to be reduced, and the  $\alpha$ -subunit to be released (Kurose *et al.*, 1983). These  $\alpha$ -subunits can activate or inhibit a number of processes (Dolphin, 1987), in this case inhibiting the effects of PAF. This theory is supported by another study in which desensitization of human platelets by platelet-activating factor was demonstrated, concluding that decreased affinity of the high affinity site was responsible for desensitization (Chesney *et al.*, 1985).

### **1.20 Desensitization as a Result of Receptor Internalisation**

Another study however (Kloprogge and Akkerman, 1984) involving rabbit platelets showed that when cells came into contact with PAF, PAF was immediately internalised. PAF receptor internalisation in washed rabbit platelets was also observed by Homma *et al.*, (1987). A study into bronchoconstriction caused by PAF in the airways of asthmatic patients also concluded that desensitization of the airways to the effects of PAF was the result of a decrease in accessible PAF receptors (Burgers *et al.*, 1993), which could be an

indication of PAF receptor internalisation. This study investigated the possibility of receptor internalisation by using immunofluorescence to label the receptors and determine whether an initial application of PAF resulted in reduced PAF binding on a subsequent addition.

### **1.21 Scope of Study & Aims**

As described at the beginning of this chapter, this study looked at the effects of PAF in the airways. The effects studied were hyperresponsiveness to ACh and 5-HT, the contractile effects of PAF itself on the airways and desensitization of the airways to the effects of PAF after an initial contact. The study used rat isolated lung strip and tracheal spiral preparations, and inhibitors of protein kinase C, lipoxygenase and phospholipase A<sub>2</sub> to investigate the possible mechanisms by which PAF has its effects. Immunofluorescence was used to localise PAF receptors within the tissues after initial and repeated PAF applications in order to try and determine any movement of the receptors (internalisation) and hence determine possible mechanisms behind desensitization (see chapter 3).

This study utilised rat isolated tracheal spirals and lung strips to compare the responses in the two situations. This enabled a comparison of effects in the larger and smaller airways. Thus in each case the following were investigated:

- The effects of PAF to cause contraction and the selective desensitization induced by PAF to PAF.
- The effect of PAF on the contractile response to ACh and 5-HT.

- Receptor internalisation.
- Whether inhibitors prevent desensitization.

## **Chapter 2**

### **Effect of PAF *per se* on the responsiveness of rat isolated lung strip and tracheal spiral to 5-HT and ACh.**

#### **2.1 Introduction**

In this study, lung strip preparations were used (as described by Lulich *et al.*, 1976) for they allow the direct investigation of drugs on the small airways of the respiratory system. The larger airways of the respiratory system are investigated using tracheal spirals (as described by Orehek *et al.*, 1975) as this method allows larger sections of tissue to remain intact than other methods such as tracheal rings. Using both tracheal spirals and lung strips, an exploration was possible for any differences in the responsiveness of the smaller and larger airways.

Previous work has shown that PAF will induce bronchoconstriction in rabbits (Pinckard *et al.*, 1982), guinea-pigs (Vargaftig *et al.*, 1980), baboons (Denjean *et al.*, 1981), humans (Cuss *et al.*, 1986) and rats (Voelkel *et al.*, 1982). This study will utilise rat trachea and lung strip preparations to investigate the PAF induced bronchoconstriction and whether PAF does selectively desensitize to PAF in these tissues.

In addition to the bronchoconstrictor properties, PAF has also been reported to increase the responsiveness of the lungs to the actions of other bronchoconstrictor agents. This increased responsiveness has been described for guinea-pig lungs (Mazzoni *et al.*, 1985; Chung *et al.*, 1986), sheep lungs (Christman *et al.*, 1987) and rat trachea (Warhurst &

Court, 1996). This study was designed to allow a comparison with the earlier studies using rat trachea and to extend the investigation with the lung strip preparations.

Thus this part of the study was to investigate the effects of PAF on the responsiveness of rat isolated lung strips and tracheal spirals to ACh and 5-HT.

In order to determine any changes in the tissues responses to exogenous agonists, following initial controls with ACh and 5-HT the responses to these agents were then determined in the presence of PAF. Once washed the tissue was exposed to a second addition of PAF and the protocol repeated. This would reveal whether the tissue was; firstly selectively desensitized to PAF; and secondly whether there was any difference in the responsiveness to 5-HT and ACh in this PAF pre-treated tissue.

## **2.2 Methods**

### **2.2.1 Tissue Preparation**

Male and female Sprague Dawley rats weighing between 250-300g were used throughout this study. Each animal was killed by stunning and bleeding, and lungs and trachea immediately removed and placed into Greenberg-Bohr buffer of the following composition (mM); NaCl, 118.9; KCl, 4.6;  $\text{KH}_2\text{PO}_4$ , 1.17;  $\text{MgSO}_4 \cdot 6\text{H}_2\text{O}$ , 1.1;  $\text{NaHCO}_3$ , 16.9; Glucose, 6.0; Sucrose, 49.9;  $\text{CaCl}_2$ , 2.1, and 0.25% (w/v) bovine serum albumin (GBB-BSA). The buffer was maintained at 37°C and gassed with 95% oxygen, 5% carbon dioxide.

### **2.2.2 Pharmacological Preparation**

Tracheal sections cut from between the larynx and main bronchi, and prepared as tracheal spirals (Tian *et al.*, 1997) and lung strips (Lopez *et al.*, 1998) of approximately 15mm long, were prepared in duplicate and suspended under 0.25g tension in GBB-BSA in 10ml organ baths. Isotonic responses were recorded using an isotonic displacement transducer (Harvard Isotonic Transducer) and a Harvard Student Oscillograph with a pre-amplifier (Harvard Freestanding Transducer Amplifier). Preparations were equilibrated for 60 min prior to addition of test agents.

Following completion of the series of experiments, the wet weights of all tissues were taken so that calculation of mm contraction per mg wet weight of tissue could be made.

### **2.2.3 Study 1 Effects of ACh and 5-HT on rat isolated tracheal spirals and lung strips**

Cumulative effects of ACh were determined for each tracheal spiral and lung strip preparation, (1nM – 1mM) and (10 $\mu$ M – 3mM) respectively. Each increase in ACh concentration was Log 0.5. The effect of each addition was confirmed as maximal prior to the application of the subsequent ACh concentration. Following the removal of ACh by washing, tissues were allowed to relax to their original baseline tensions and equilibrated for 30 min prior to the addition of further agents (four ACh concentration effect curves in total were performed on each individual tissue preparation).

Before each of the following three ACh concentration effect curves were performed, the effects of single doses of 5-HT (30  $\mu$ M) were determined on one of each type of tissue preparation, with a contact time of 18 min. A concentration of 30  $\mu$ M was chosen as this had been found to produce optimal tissue responses in previous work within this laboratory (Warhurst & Court, 1996). For other preparations of each tissue type, ascorbic acid (2.8  $\mu$ M) was used to act as a solvent matched control against the 5-HT.

After each ACh concentration effect curve had been performed, tissues were washed, and once returned to baseline, equilibrated for 30 min prior to the addition of further agents.



#### **2.2.4 Study 2 Effects of PAF *per se* on responses to ACh and 5-HT**

Four ACh concentration effect curves in total were performed on each individual tissue preparation. The initial two ACh concentration effect curves were performed as in 2.2.3, initially with ACh alone, and then following after application of either 5-HT or ascorbic acid.

Before the third and fourth curves were performed, tissues were allowed 30 min equilibration after returning to baseline. The effects of single doses of PAF (1 $\mu$ M) were then determined for each preparation using a contact time of 3 min. The effects of 5-HT (30 $\mu$ M) were then determined with contact times of 15 min before the addition of ACh. After ACh concentration effect curves had been performed, tissue preparations were washed and, once returned to baseline, equilibrated for 30 min. To control tissues ascorbic acid (2.8  $\mu$ M) was added in place of the 5-HT.

#### **2.2.5 Statistical Analysis of Results**

Results from the pharmacological study were compared to their controls using a one way ANOVA (analysis of variance). A Tukey family error rate of 0.05 was used and p values of less than 0.05 were taken as indicating a significant difference. The Tukey test is a post-hoc multiple comparison repeated measures test. It allows statistical comparisons to be made between the means of each population, taking into account the individual values that are used to calculate the means. The statistical package 'MINITAB' was used for the statistical analysis.

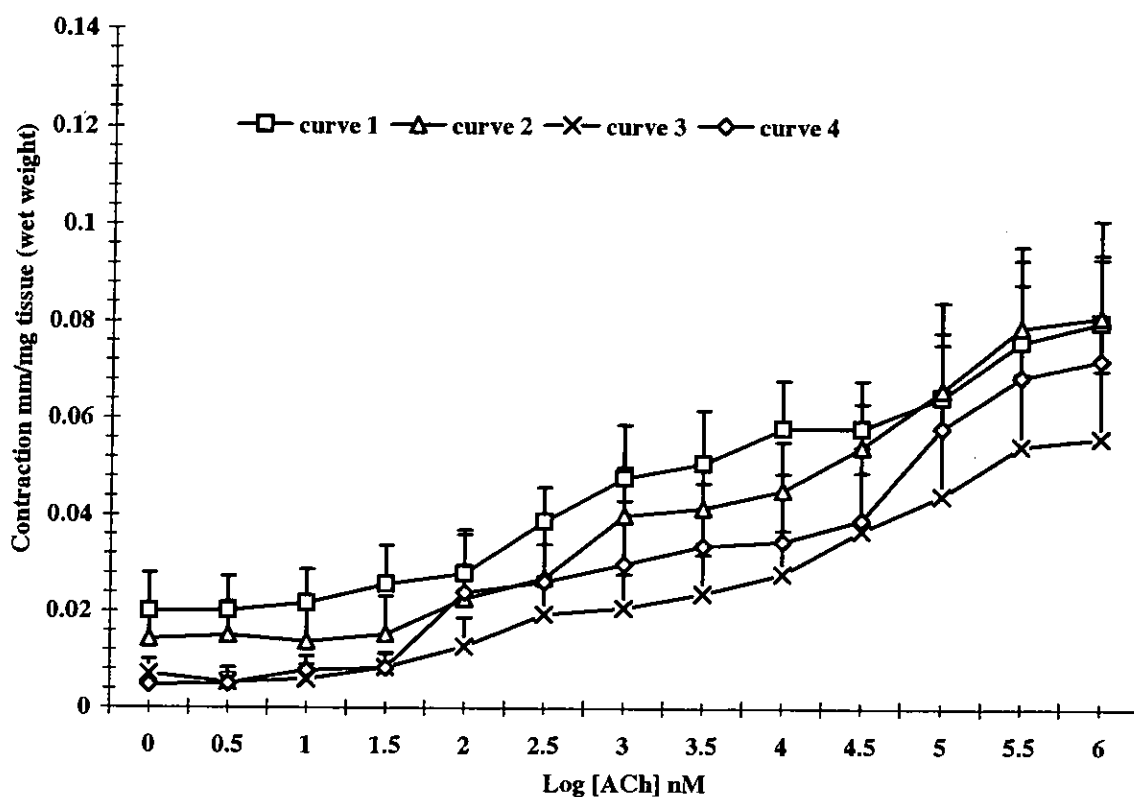
## **2.3 Results**

### **2.3.1 Study 1 Effects of ACh and 5-HT on rat isolated tracheal spirals and lung strips**

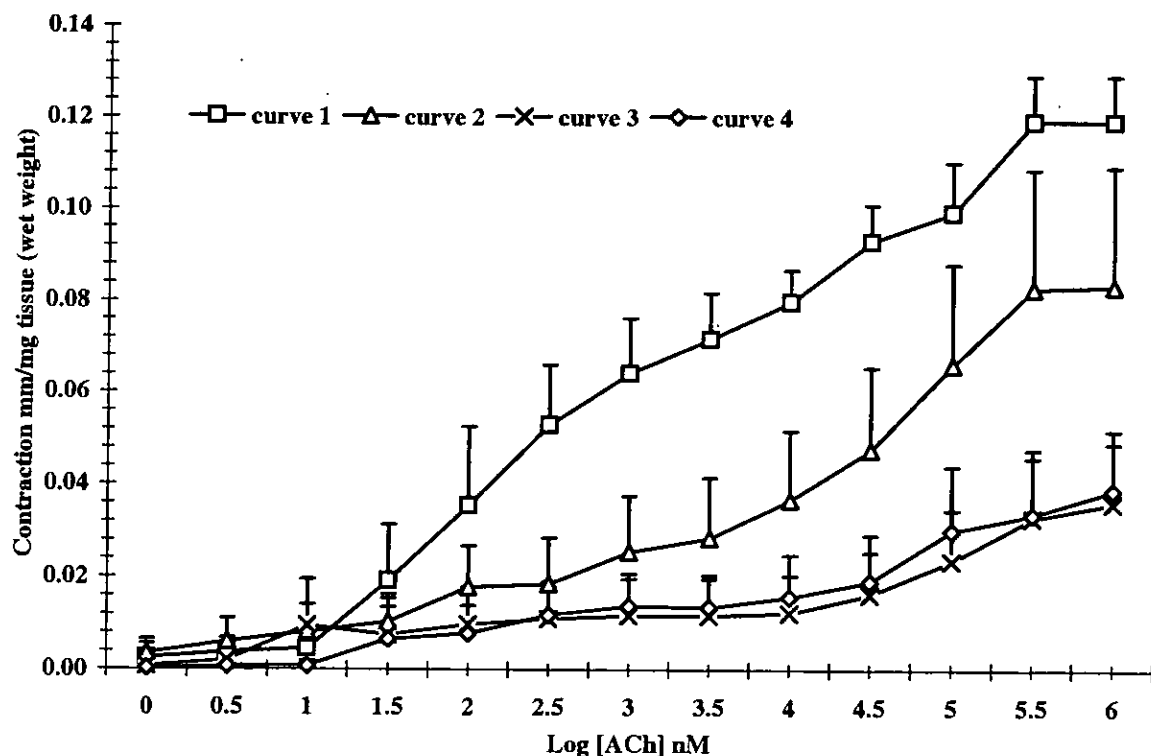
ACh produced a concentration-dependent contraction of both the trachea and lung strip preparations. The repeated concentration effect curves given by tracheal spirals following cumulative addition of ACh in the presence of either 5-HT or ascorbic acid are shown in figures 4 and 5 respectively. There were no significant differences between the concentration-effect curves to ACh obtained in the presence of 5-HT and those obtained in the presence of ascorbic acid. This indicated that 5-HT was not causing increased responsiveness to ACh.

The repeated concentration effect curves given by lung strips after cumulative addition of ACh in the presence of either 5-HT or ascorbic acid are shown in figures 6 and 7 respectively. Again, no significant differences occurred between the responses obtained after addition of 5-HT and those given in the presence of ascorbic acid.

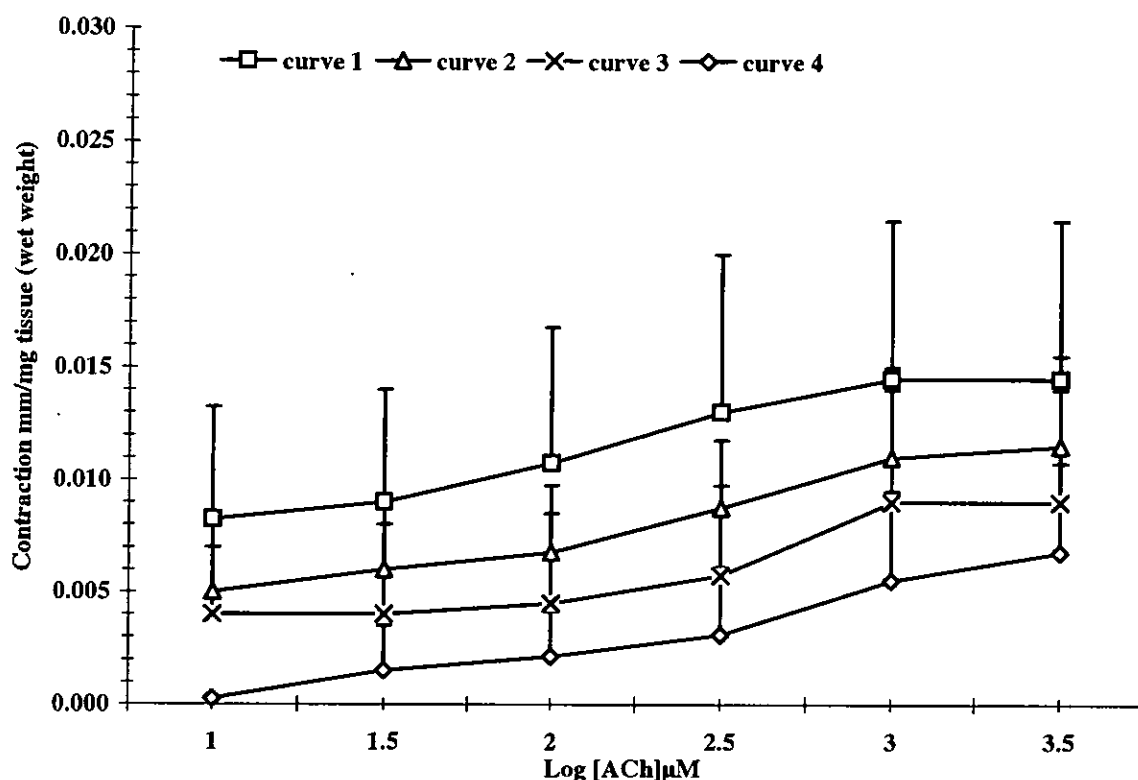
5-HT contracted both the trachea and lung strip of the rat. The effects of the repeated additions of 5-HT on the trachea and on the lung strip are shown in figures 8 and 9. A decline in the responses of both tissues to 5-HT was evident with subsequent 5-HT additions. In the trachea, this decline reached statistical significance between the first and third responses ( $p = 0.02$  ANOVA). However, lung strip responses failed to reach statistical significance.



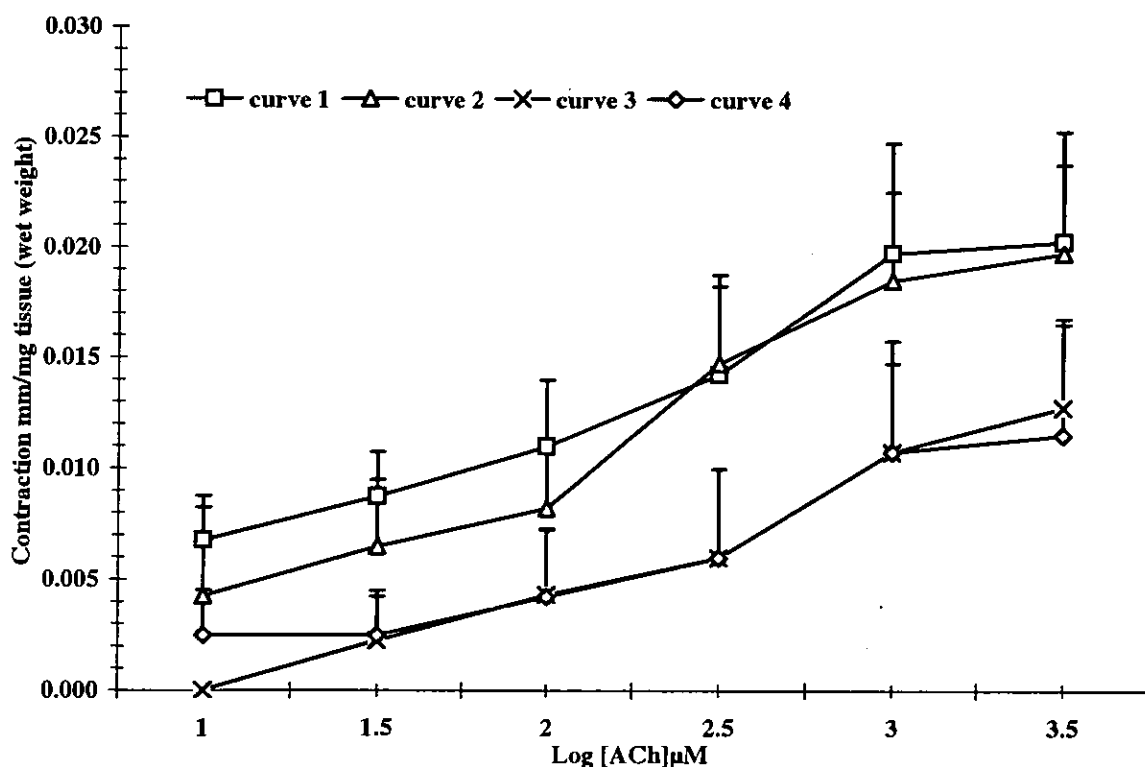
**Fig. 4 Repeated ACh concentration effect curves on rat isolated tracheal spirals.**  
 Curve 1, ACh alone; Curve 2, with 1'st addition of 5-HT; Curve 3, 2'nd 5-HT addition;  
 Curve 4, 3'rd 5-HT. Values expressed as mean  $\pm$  S. E. M., n=4



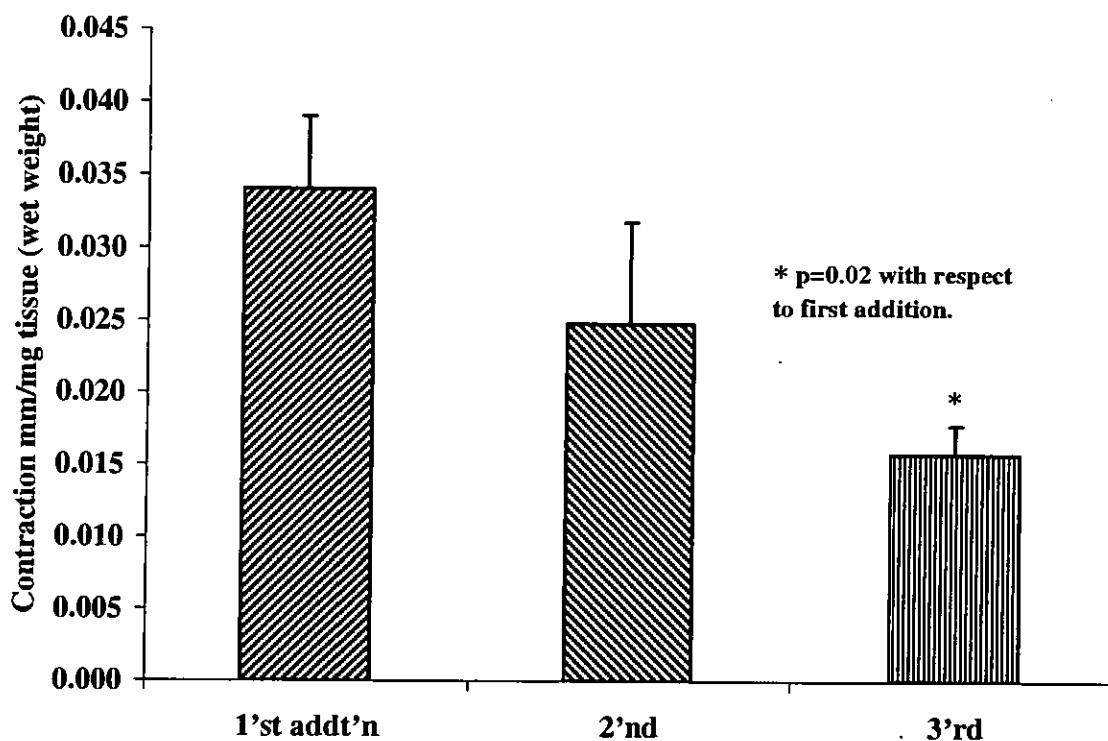
**Fig. 5 Repeated ACh concentration effect curves on rat isolated tracheal spirals.**  
 Curve 1, ACh alone; Curve 2, with 1'st addition of ascorbic acid (aa); Curve 3, 2'nd aa  
 addition; Curve 4, 3'rd aa. Values expressed as mean  $\pm$  S. E. M., n=4



**Fig. 6 Repeated ACh concentration effect curves on rat isolated lung strips.**  
 Curve 1, ACh alone; Curve 2, with 1'st addition of 5-HT; Curve 3, 2'nd 5-HT addition  
 Curve 4, 3'rd 5-HT. Values expressed as mean  $\pm$  S. E. M., n=4

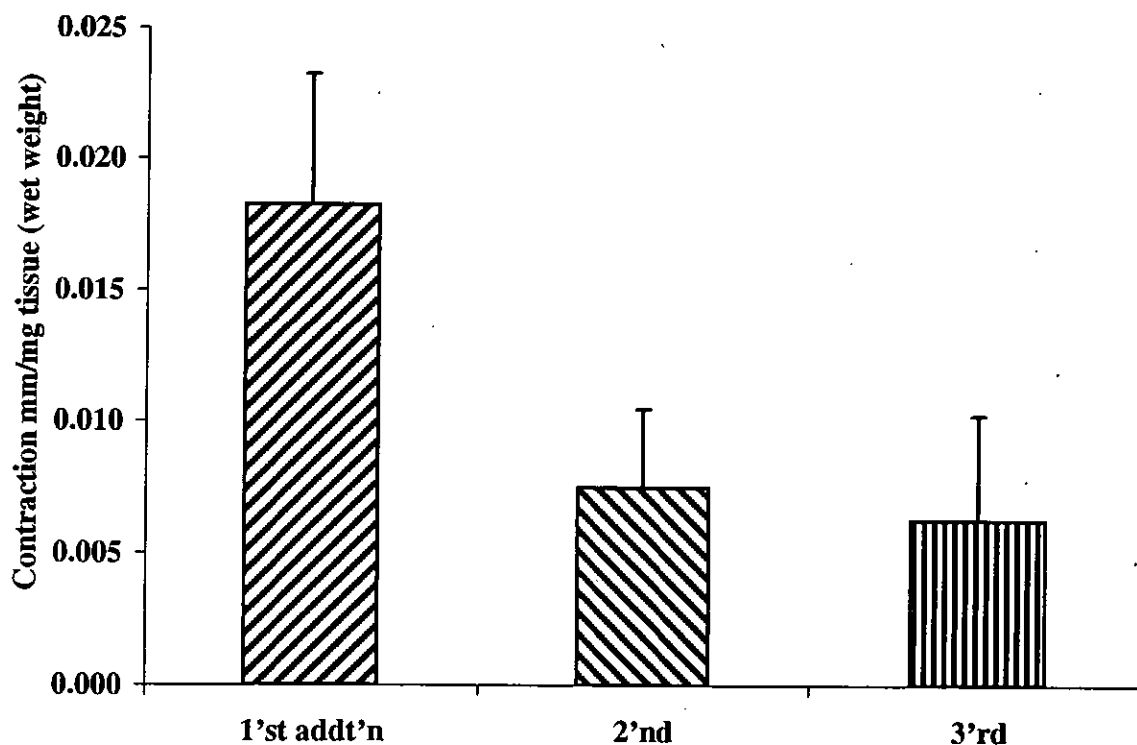


**Fig. 7 Repeated ACh concentration effect curves on rat isolated lung strips.**  
 Curve 1, ACh alone; Curve 2, with 1'st addition of ascorbic acid (aa); Curve 3, 2'nd a  
 addition; Curve 4, 3'rd aa. Values expressed as mean  $\pm$  S. E. M., n=4



**Fig. 8 Repeated 5-HT induced contractions in rat isolated tracheal spirals.**

1 indicates response on first addition, 2 the second addition, and 3 third  
(Values expressed as mean  $\pm$  S. E.M., n=4).



**Fig. 9 Repeated 5-HT induced contractions in rat isolated lung strips.**

1 indicates response on first addition, 2 the second addition, and 3 third.  
(Values expressed as mean  $\pm$  S. E.M., n=4).

### **2.3.2 Study 2 Effects of PAF *per se* on responses to ACh and 5-HT**

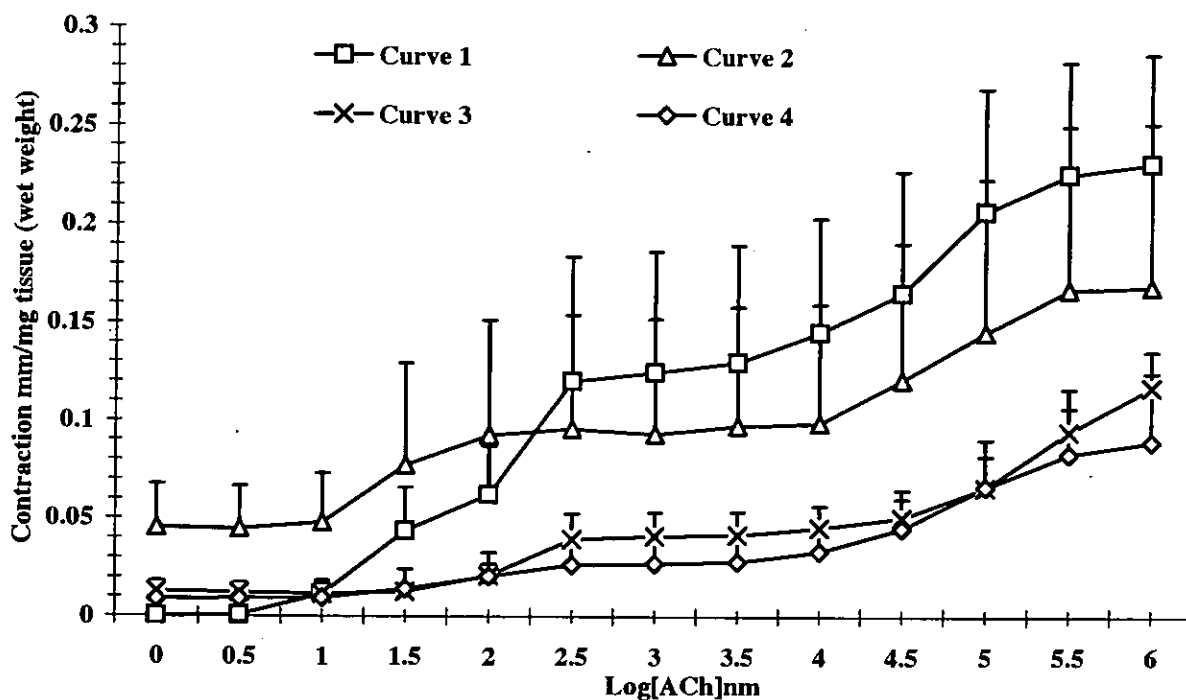
The repeated concentration effect curves given by tracheal spirals on the cumulative addition of ACh in the presence of either PAF and 5-HT, or PAF and ascorbic acid are shown in figures 10 and 11 respectively. Significant differences ( $p \leq 0.05$ ) occurred between the ACh curves performed after one PAF application with 5-HT (see fig. 10), and its control. The responses to ACh were significantly higher in the tissues that had received 5-HT, this appeared to be due mainly to the 5-HT partially contracting the tissue thus giving an artificially high baseline which gave an apparent increase in ACh responsiveness (as measured from the baseline prior to 5-HT addition).

Figures 12 and 13 show repeated ACh concentration effect curves given by lung strips on the cumulative addition of ACh in the presence of either 5-HT (figure 12), or ascorbic acid (figure 13). There were no statistically significant differences between the results obtained in the presence of either 5-HT or ascorbic acid. Lung strips are not ideal tissues to use due to the massive variability in the contractility and the small responses given by the tissue.

No hyperresponsiveness to ACh was observed with the tracheal spiral preparations in the presence of PAF. Though, in the lung strips, where 5-HT was present, a first addition of PAF did effect some hyperresponsiveness to ACh (figure 12), although this did not reach statistical significance. Hyperresponsiveness was not observed in the presence of the second PAF addition.

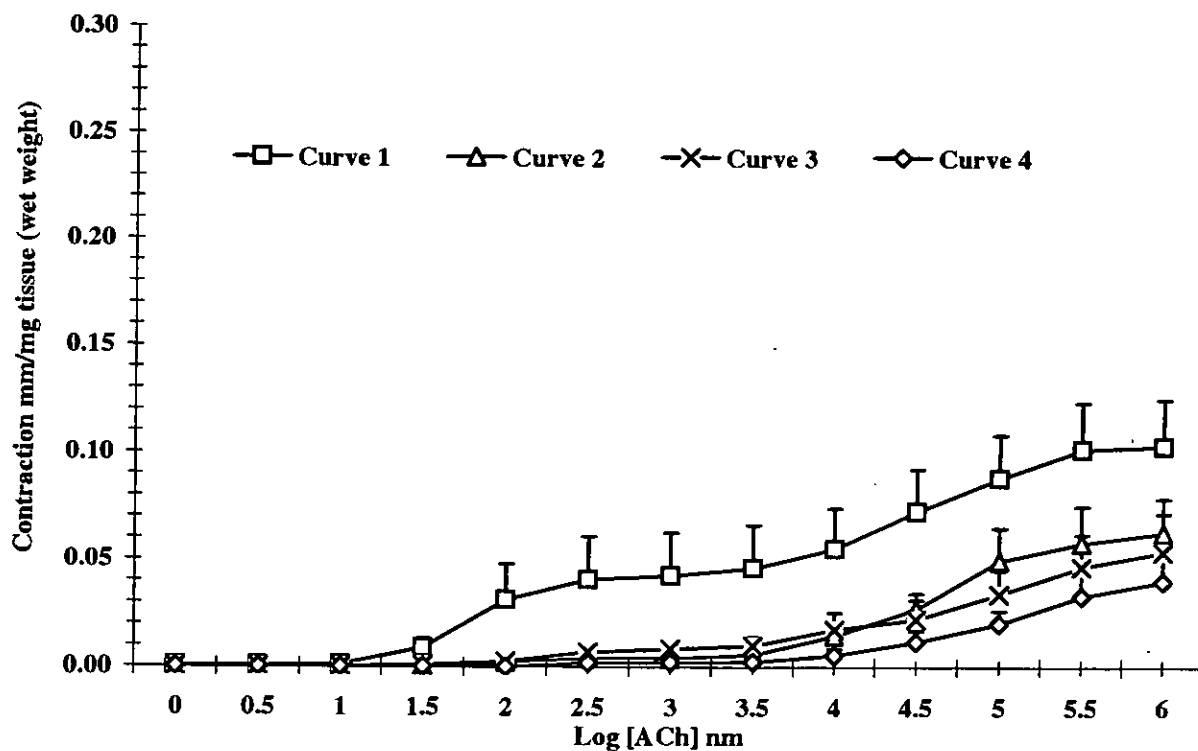
Tracheal contractions following repeated additions of 5-HT in the presence of PAF compared to repeated additions of 5-HT alone are seen in figure 14. PAF had no effect upon the actions of 5-HT, and no statistical differences were found between the results obtained in the presence of PAF and relevant controls. Similar results for the lung strip were recorded (figure 16), and no significant differences were found between contractions.

Figures 15 and 17 represent responses given by tracheal spirals and lung strips respectively, following the addition of two separate doses of PAF. In the preparations of both tissue types that were to receive 5-HT, when PAF was added for the first time, tissue contraction was observed. However, on second addition of PAF no further contractions occurred. These observations are supported statistically ( $p = 0.039$  and  $p = 0.007$  for the trachea and lung strip respectively). Throughout the study ascorbic acid had no effect on any of the tissue.



**Fig. 10 Repeated ACh concentration effect curves on rat isolated tracheal spirals.**

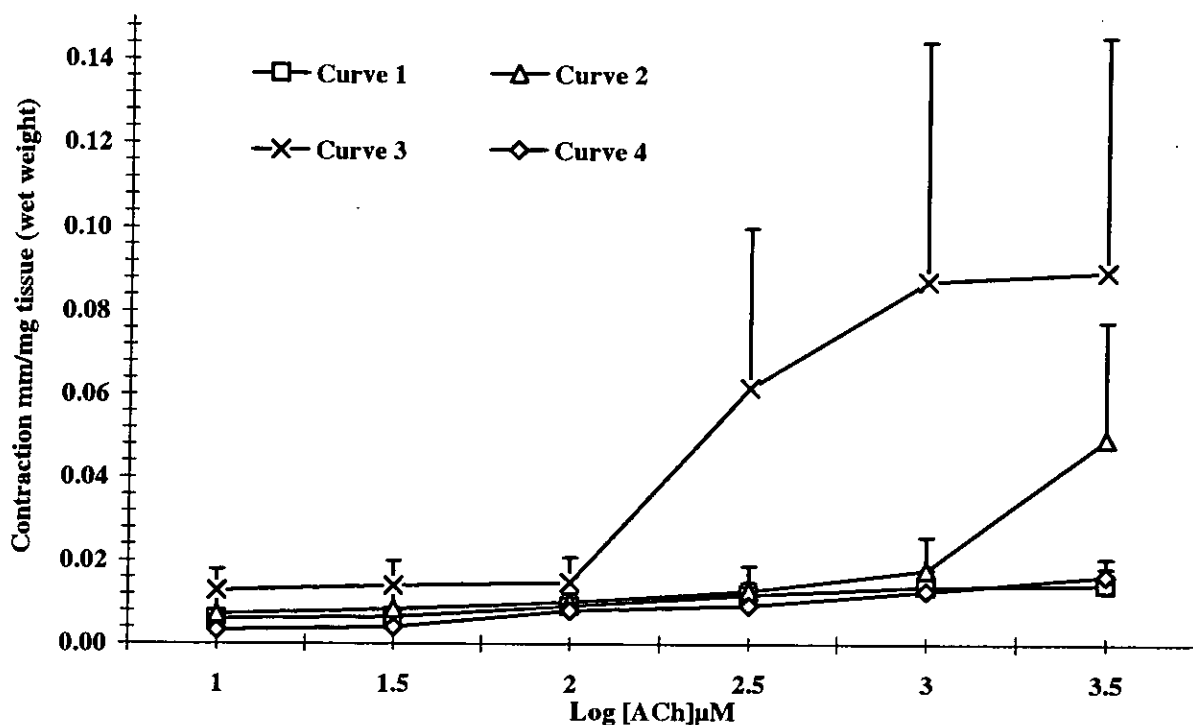
Curve 1, ACh alone; Curve 2, with 5-HT; Curve 3, with PAF and 5-HT; Curve 4, with PAF and 5-HT again. Values expressed as mean  $\pm$  S. E. M.,



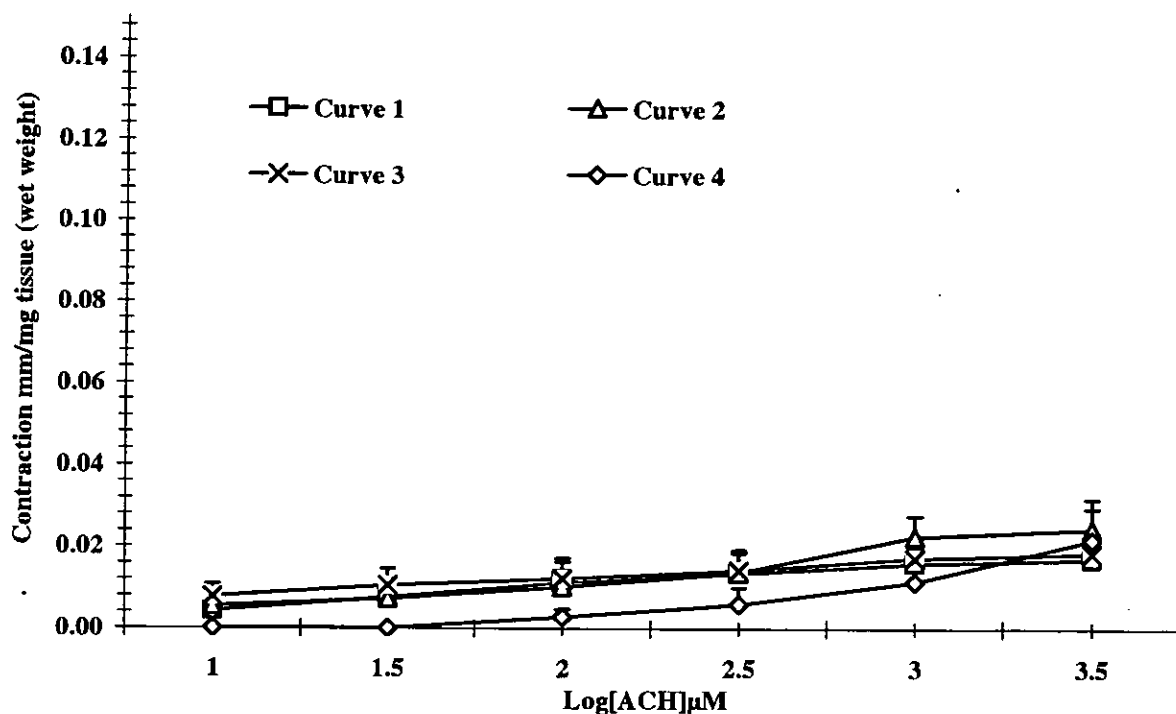
**Fig. 11 Repeated ACh concentration effect curves on rat isolated tracheal spirals.**

Curve 1, ACh alone; Curve 2, with ascorbic acid (aa); Curve 3, with PAF and aa; Curve 4, with PAF and aa again. Values expressed as mean  $\pm$  S. E. M., n=4

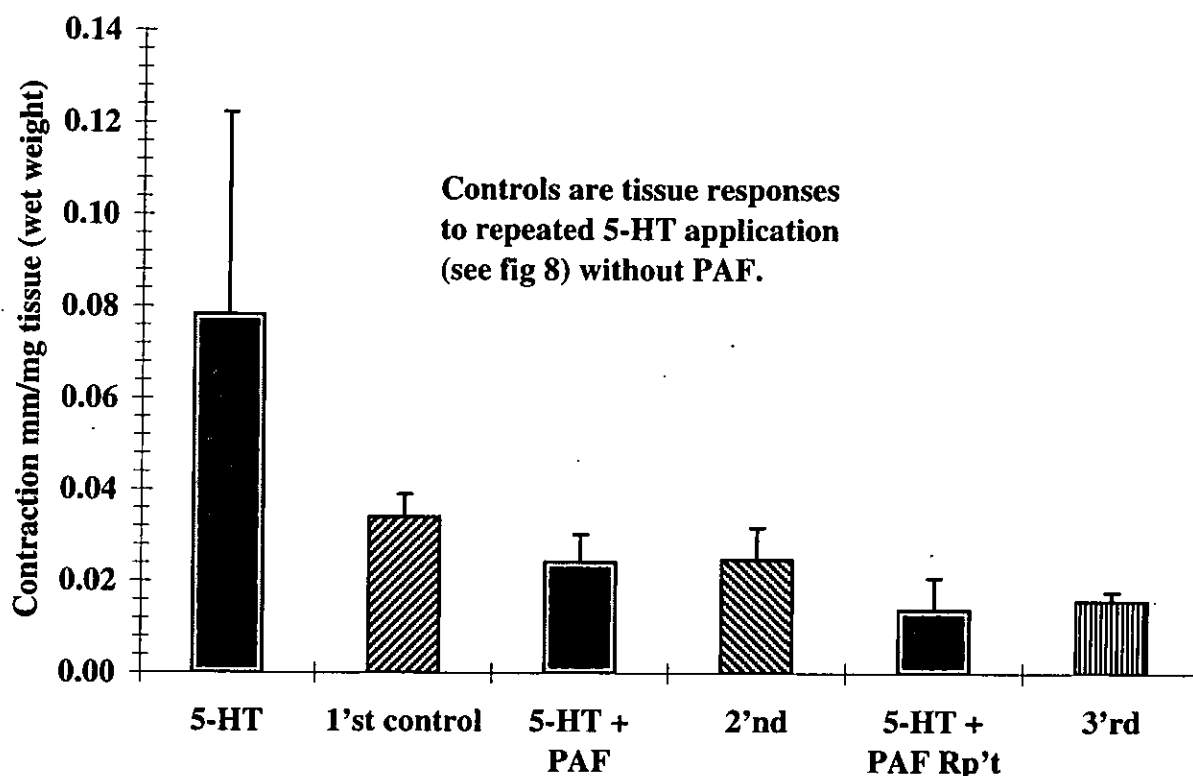




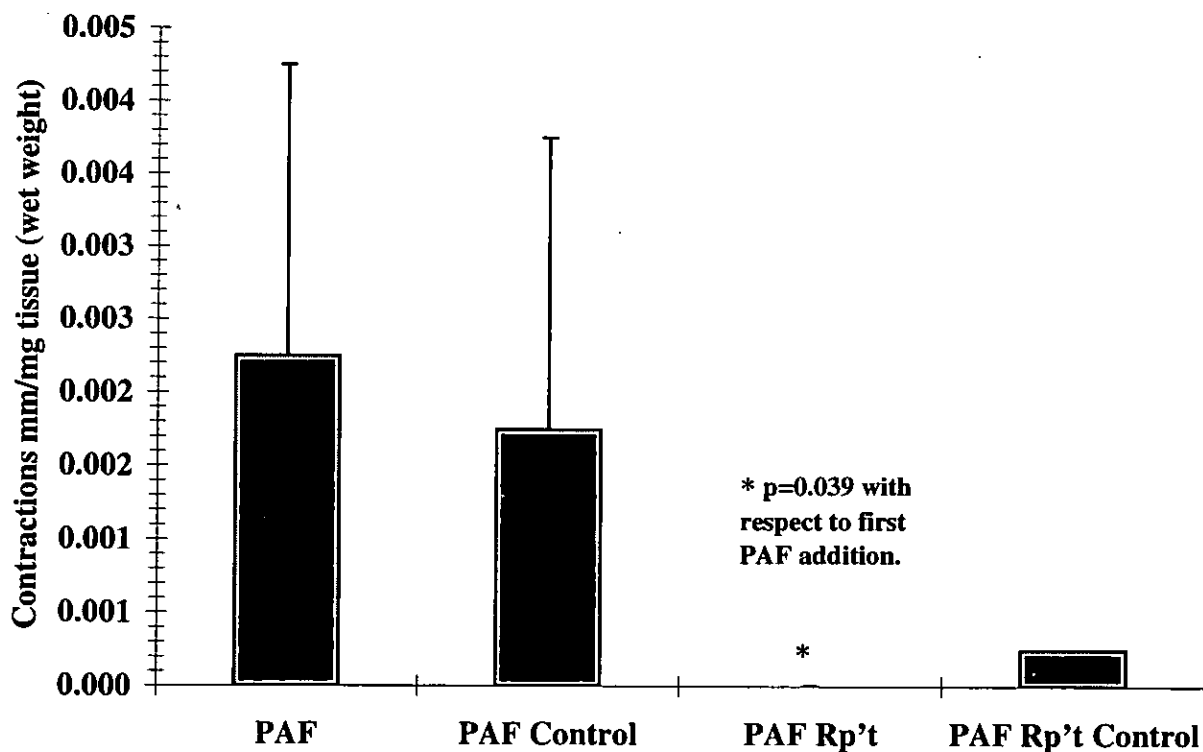
**Fig. 12 Repeated ACh concentration effect curves on rat isolated lung strips.**  
 Curve 1, ACh alone; Curve 2, with 5-HT; Curve 3, with PAF and 5-HT; Curve 4, with  
 PAF and 5-HT again. Values expressed as mean  $\pm$  S. E. M., n=4



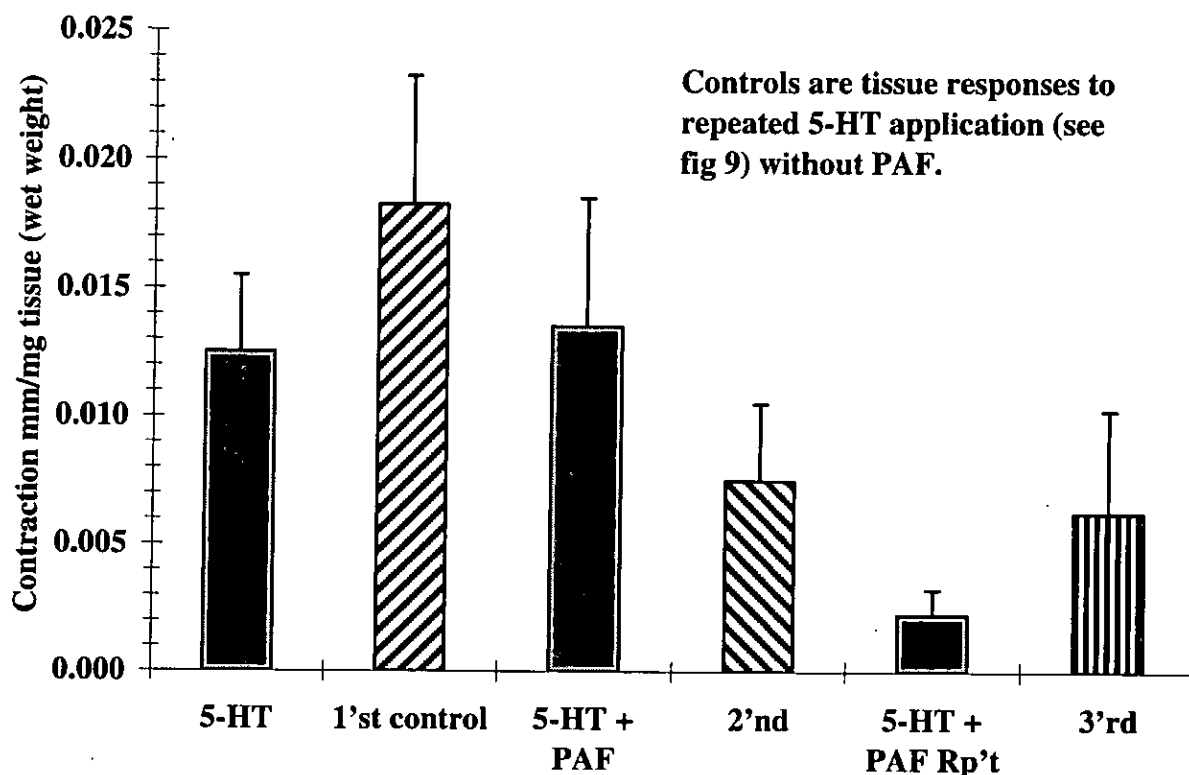
**Fig. 13 Repeated ACh concentration effect curves on rat isolated lung strips.**  
 Curve 1, ACh alone; Curve 2, with ascorbic acid (aa); Curve 3, with PAF and aa;  
 Curve 4, with PAF and aa again. Values expressed as mean  $\pm$  S. E. M., n=4



**Fig. 14 Responses given by tracheal spirals on repeated addition of 5-HT in the presence and absence of PAF.**  
Values expressed as mean  $\pm$  S. E. M., n=4.

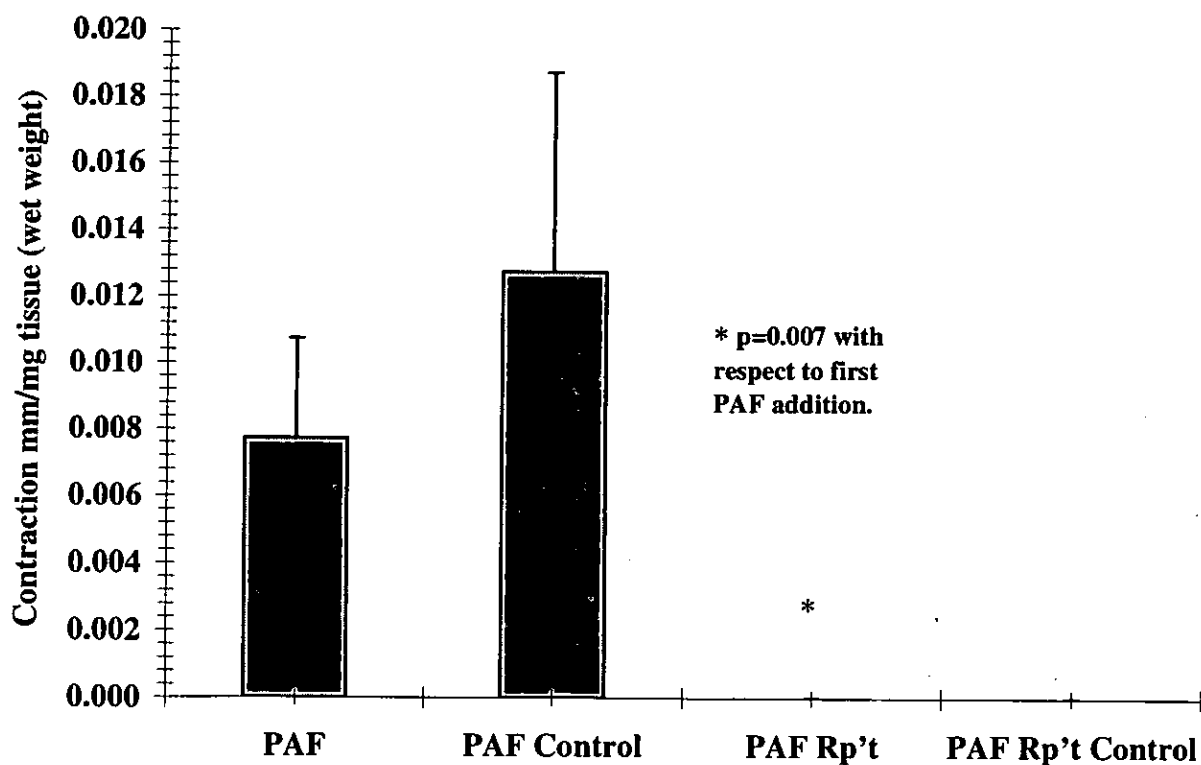


**Fig. 15 Responses given by tracheal spirals in response to PAF.** (PAF represents tissue which received 5-HT and Control represents tissue which received ascorbic acid). Values expressed as mean  $\pm$  S. E. M., n=4.



**Fig. 16 Responses given by lung strips on repeated addition of 5-HT in the presence and absence of PAF.**

Values expressed as mean  $\pm$  S. E. M.,  $n=4$ .



**Fig. 17 Responses given by lung strips in response to PAF.**

(PAF represents tissue which received 5-HT and Control represents tissue which received ascorbic acid). Values expressed as mean  $\pm$  S. E. M.,  $n=4$ .

## **2.4 Discussion**

### **2.4.1 Study 1 Effects of ACh and 5-HT on rat isolated tracheal spirals and lung strips**

In this part of the investigation, comparison of results with ascorbic acid controls showed that the presence of 5-HT was having no effect on the contractions given by tissues in response to ACh application. Although the differences did not reach significance, the results showed that the size of contractions in both lung strips and tracheal spirals became smaller with repetition of ACh concentration effect curves (see figures 4-7). This decrease in response was also seen in the contractions given by both tracheal spirals and lung strips with repeated 5-HT addition (see figures 8 and 9). Difficulties in proving the results statistically significant appeared to be due to large standard errors of the means.

These decreases in sizes of contractions are most likely due simply to muscle fatigue, and in the case of the 5-HT responses, the results act as controls against which to compare the effects of drugs throughout the study.

### **2.4.2 Study 2 Effects of PAF on responses to ACh and 5-HT**

Hyperresponsiveness, to ACh in the presence of PAF, as was observed in previous work (Warhurst and Court, 1996) on tracheal spirals was not observed in this study (see figures 10 and 11). Hyperresponsiveness was also not observed in lung strip preparations (see figures 12 and 13). In tracheal spiral preparations, the second ACh concentration effect curve performed, in the presence of 5-HT was significantly higher than its control with ascorbic acid. This was due however to tissues not properly returning to baseline tension

after addition of 5-HT and before addition of ACh. PAF had no effect upon the tissue contractions given in response to 5-HT in either tracheal or lung strip preparations (see figures 14 and 16 respectively).

The lack of observed PAF induced tissue hyperresponsiveness observed, could be the result of either; a) it not occurring, thus conflicting with the findings of previous studies, b) other factors such as blood present in lung tissue causing variability in results or c) rats coming from a different source to those used in other studies and responding differently to the experimental procedures.

### **2.4.3 Effects of PAF *per se***

The addition of PAF itself brought about contractions in both tracheal and lung strip preparations (see figures 15 and 17 respectively). These contractions, seen in tissues which had received 5-HT previously, and those which had received ascorbic acid, were not seen after subsequent PAF additions indicating desensitization of the tissues to the effects of PAF. In control tracheal preparations which had received ascorbic acid previously, a small contraction was recorded after the second PAF addition, but this was the result of one preparation from the four which make up the results not quite returning to baseline tension after the previous ACh concentration effect curve. This desensitization of tissue to the effects of PAF is supported by previous work on guinea pig lung tissue (Honda *et al.*, 1991). The desensitization induced by PAF in this study is homologous desensitization, as the responses to ACh and 5-HT were not effected, despite PAF no longer producing a response.

It is worth noting that the trends observed for both of tracheal and lung tissue in response to PAF, and to other drugs were very similar, although the actual size of the contractions produced by tracheal tissue (mm/mg tissue) were much larger than those produced by lung tissue. This could be due to a higher proportion of lung tissues mass being made up of cells other than muscle cells such as blood cells.

Once again high standard error of mean values (up to 0.057 in lung strip) were observed, the reason for which had to be determined as it may have been affecting the tissues responses to PAF and other drugs used. It was observed when dissecting tissues from rats that there were varying amounts of blood within the chest cavity as a result of the method of killing the animals, which often made its way into the lung tissue. This introduces the possibility of the results being affected by eosinophils or other blood cells which became lodged within the lungs. These cells could be activated by the addition of exogenous PAF so releasing mediators which may influence the size of the contractile response in lung tissue. Indeed Gleich *et al* (1988) found that eosinophils became activated by PAF and caused an increase in the responsiveness of lung tissue. Also, Vargaftig *et al* (1980) found that PAF induced a platelet-dependent bronchoconstriction unrelated to the formation of eicosanoids.

This does not however explain the variability in the lungs responsiveness to ACh and 5-HT, though an attempt was made to reduce variability by expressing the results as mg wet weight tissue.

## **Chapter 3**

### **Investigation into PAF receptor internalisation and possible presence and effect of eosinophils.**

#### **3.1 Introduction**

Previous studies have suggested that PAF receptor internalisation may be responsible for the desensitization of airway tissue to the effects of PAF (Kloprogge and Akkerman, 1984). This part of the study attempted to localize PAF receptors within the tissues using immunofluorescence (IF) techniques. Subsequently, studies investigated PAF receptor IF to determine the localization of PAF receptors in tissues subjected to repeated exposure to PAF.

The large variation in results obtained in the pharmacological investigation (chapter 2), indicated that other variables may have influenced the data obtained. It was also noted, when dissecting tissue from the rats, that blood was often present in lung tissue, and had sometimes passed down the trachea. One property of PAF is its potent activity as a chemotactic agent and as an activator of eosinophils, which are prominent cells in asthmatic airways (Chung and Barnes, 1991). It was therefore decided that as airway hyperresponsiveness has been linked to eosinophilic inflammation in the airways (Gleich *et al.*, 1998), and that specific binding sites for PAF are found on eosinophils (Ukena *et al.*, 1989), the possibility of eosinophils affecting the results should be investigated. This was also done with the use of an IF method.

Immunocytochemistry (ICC) was the principle method used in this part of the study. ICC allows cell by cell resolution, the identification of individual cells, and determination of the exact localization, distribution and density of the antigen (in this case PAF-receptor).

ICC studies use antibodies which are employed for the localization of proteins or peptides in their normal cellular environment (Griffiths, 1993).

To achieve this, a number of procedures were carried out. The tissue had to be fixed to maintain the protein or peptide of interest in its normal position and prevent it from being degraded. The next stage was the selection of an antiserum specific for the antigen in question. The antiserum had to be 'pure' otherwise background staining would have occurred. The timing of incubation, temperature and dilution of the primary antibodies were crucial factors which had to be determined in order to carry out the ICC. The variable region of the antibody binds on the antigen of interest leaving its constant region exposed. The secondary antibody, which is conjugated with fluorescent molecules, in this study either FITC (green) or CY3 (red) fluorescent molecules, binds to the constant region. It is important to note that in this study, an IF technique was used, and not an enzyme-substrate based ICC technique.

The purpose of chemical fixation is to retain the antigenicity of the component to be labeled, in order that the primary antibody attaches to the antigen leading to positive staining. 'Overfixation' on the other hand, will prevent the antigen from being bound by the antibody. The nature of the fixative chosen depends upon the chemistry of the



antigen. For example a fixative containing alcohol cannot be used to fix a peptide antigen, since many peptides can be extracted from tissues using alcohols. The tissues used in this study were fixed with cold acetone.

The aims of this part of the study were therefore; to determine the effect of PAF exposure on the localization of PAF receptors in lung and tracheal tissue; to confirm the presence of any eosinophils, and to identify any PAF receptors on these eosinophils.

## **3.2 Materials and Methods**

### **Study 3 Immunofluorescent (IF) investigation into the localization of PAF receptors in the trachea and lung strip and the effect of PAF upon their localization.**

For all IF investigation, four tissues/slides were produced.

#### **3.2.1 Preparation of Tissue**

Lung strips and sections of trachea (not tracheal spirals) were prepared and incubated in buffer before being treated with PAF, as previously (chapter 2). 5mm sections of each tissue type were removed at various stages in the treatment (before PAF treatment, after one PAF treatment and after two PAF treatments), and immediately frozen in isopentane cooled with dry ice, before being wrapped in parafilm and stored at -80°C.

Tissue was collected at the following stages in the experiment; a) Immediately after removal from the rat, b) After the first 30 minute incubation with PAF and c) After the second 30 minute incubation with PAF.

Subsequently, 16µm cryostat sections were cut, mounted on 0.5% chrome-alum-gelatine coated slides, and heat fixed for approximately 5 min before being subjected to IF.

#### **3.2.2 Immunofluorescent (IF) Procedure**

The procedures followed for the IF method adopted were as follows:

Sections were fixed in cold acetone for 10 min before being washed 3 times in phosphate buffered saline (5 min each time).

Sections were incubated for 20 min with 10% normal rabbit serum.

Following washing (as before), sections were incubated overnight at 4°C with either primary antibody (see table 3) or control serum in PBS with 1.5% normal rabbit serum.

After washing, the slides were then incubated for 4 hours at 4°C in the dark with a conjugated secondary antibody (see table 1) in PBS with 1.5% normal rabbit serum.

Finally, the tissues were washed, cover slipped and viewed under a fluorescent light microscope using an appropriate filter.

### 3.2.3 Imaging technique

Receptor staining was observed with a Leitz Diaplan Photomicroscope equipped with FITC (green) and CY3 (red) optics. The process of imaging immunofluorescence staining was achieved with the aid of a computerized package called SIRIUS (optivision). This refined the images that were taken with the help of a SONY colour video camera / CCD-IRIS attached to the fluorescent microscope

**Table 1. Primary and secondary antibodies with concentrations**

What stained for	Primary antibody	Control Serum	Concentration	Secondary antibody	Concentration
PAF-receptors	anti-PAF	Normal goat serum	1:50	Anti-goat IgG with FITC conjugate	1:500
Eosinophils, basophils, and mast cells.	anti-human-CD40 ligand	Normal goat serum	1:25	anti-mouse CY3	1:500

### **3.2.4 Study 4 Double Immunofluorescence (IF) of eosinophils and PAF receptors.**

In order to co-localise PAF receptors and eosinophils, a protocol was developed so as to allow double IF staining. The protocol adopted was as follows:

Protocol was followed similarly as in study 3. Sections treated with anti-PAF were incubated overnight at 4°C in the dark with 1:25 anti-human CD40 with 1.5% normal rabbit serum in PBS. For controls, anti-human CD40 was replaced by normal goat serum.

Washed sections were subsequently incubated for a further 4 hours at 4°C in the dark with 1:500 anti-mouse CY3.

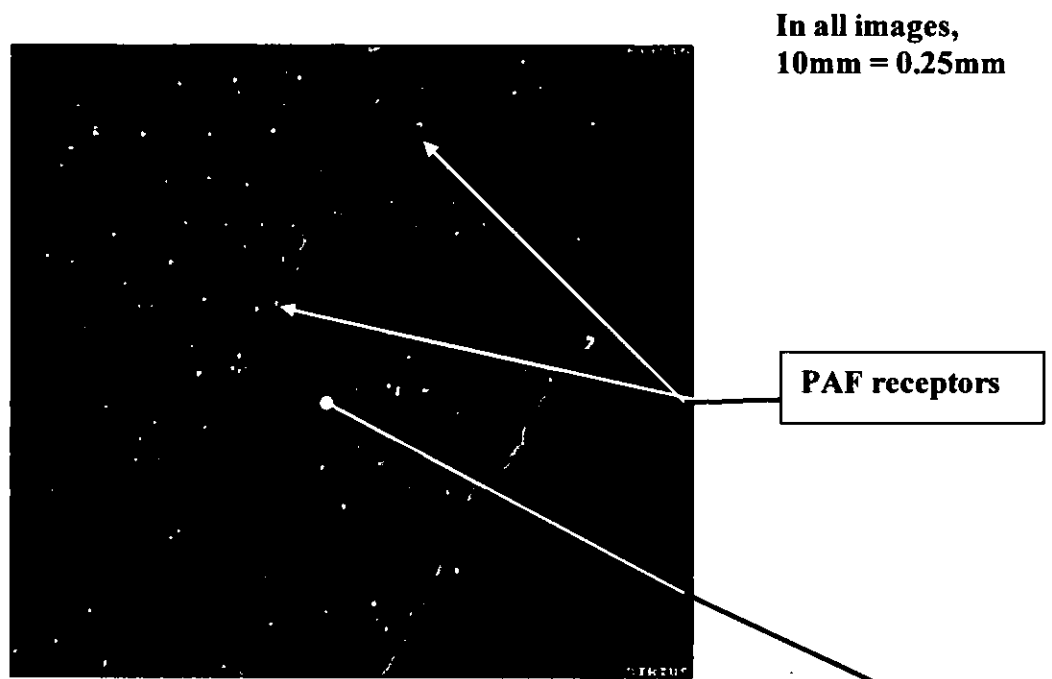
Sections were then viewed as before.

### **3.3 Results**

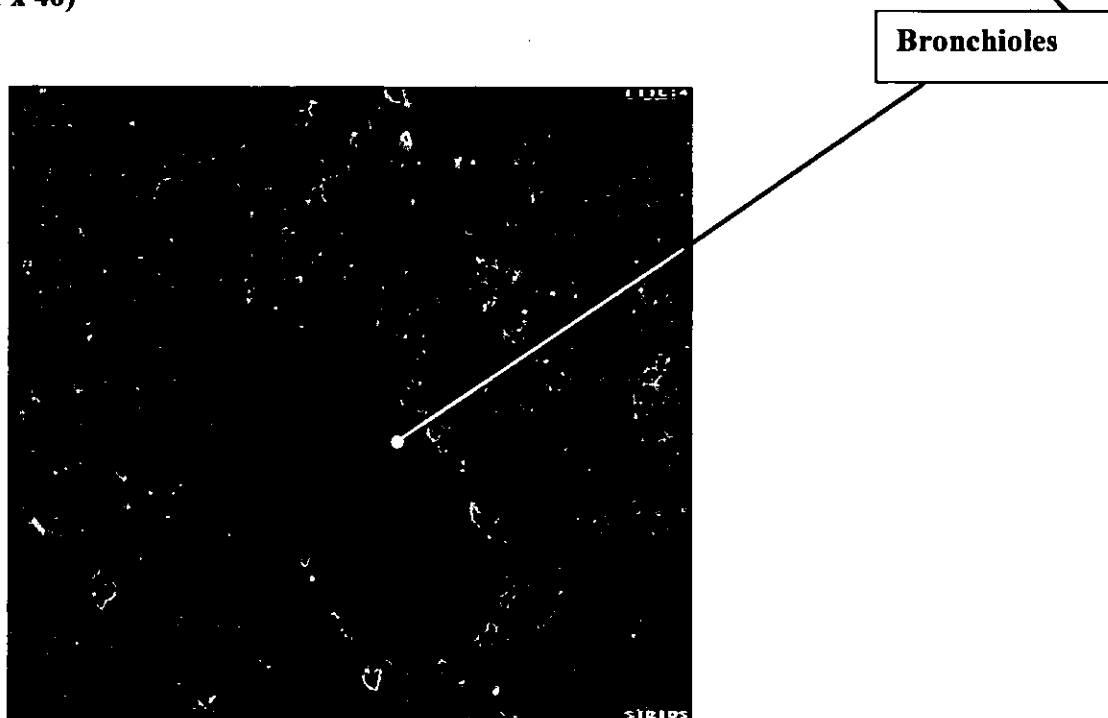
#### **3.3.1 Study 3 Immunofluorescent (IF) investigation into the localization of PAF receptors in the trachea and lung strip and the effect of PAF upon their localization.**

Figure 18 shows a typical slide of PAF receptor immunofluorescence in the lung strip before the addition of PAF. PAF receptors appear as bright green IF. Slight fluorescence is present predominantly around the bronchioles. Figure 19 shows the control for figure 18, where the primary antibody, anti-PAF, was replaced with normal goat serum. Figure 19, which shows considerably less IF, provides further evidence any that staining in figure 18 is specific for PAF receptors. For each IF study performed, simultaneous controls were used. These controls consistently produced very little fluorescence.

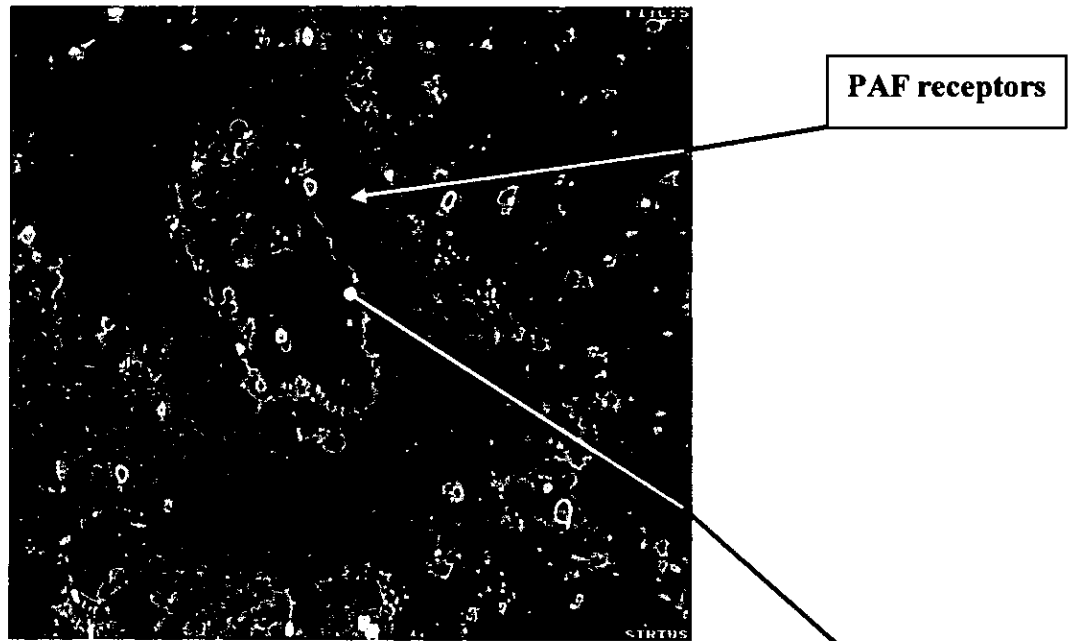
Figures 20 and 21 show sections from lung strip (from same animal as in figures 18 and 19), after being treated with PAF and after being exposed to PAF twice, respectively. No significant differences in the localization or density of PAF receptor IF were evident. Figures 22 and 23 show sections of trachea from the same animal. Figure 22 shows PAF receptors after one addition of PAF, whilst figure 23 shows PAF receptors after two PAF exposures. As in the lung tissue, repeated exposure to PAF had no effect on the density or localization of PAF receptor IF.



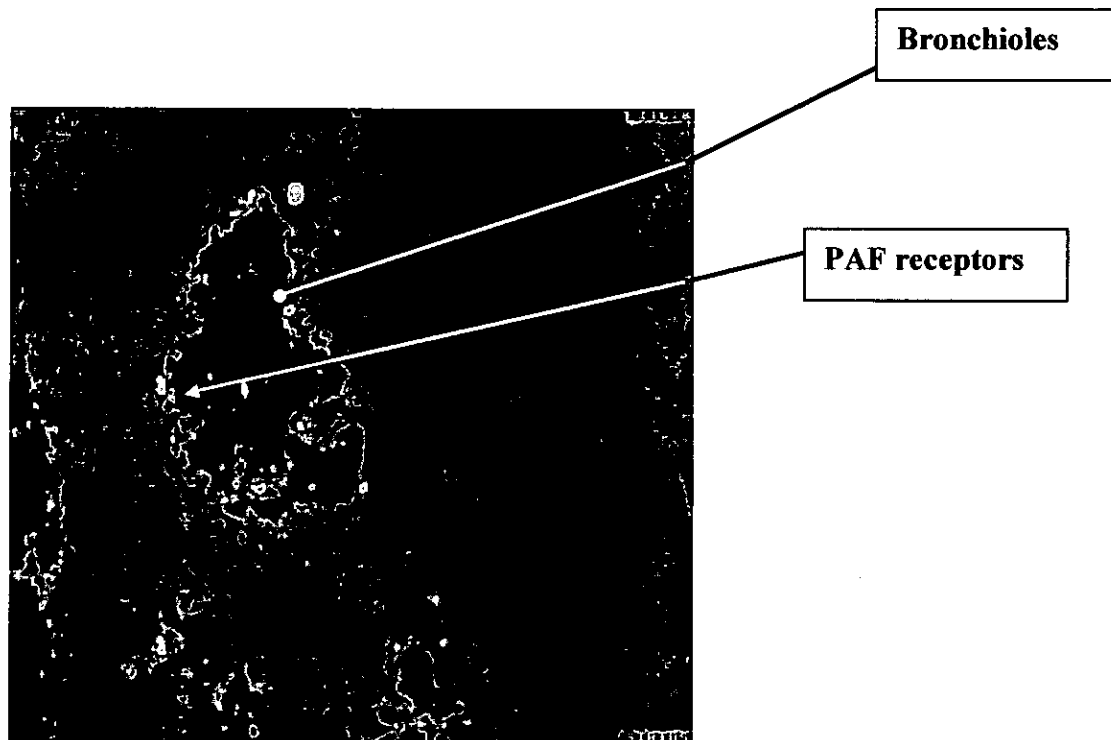
**Figure 18. PAF receptor IF in the lung strip before exposure to PAF.**  
(Magnification x 40)



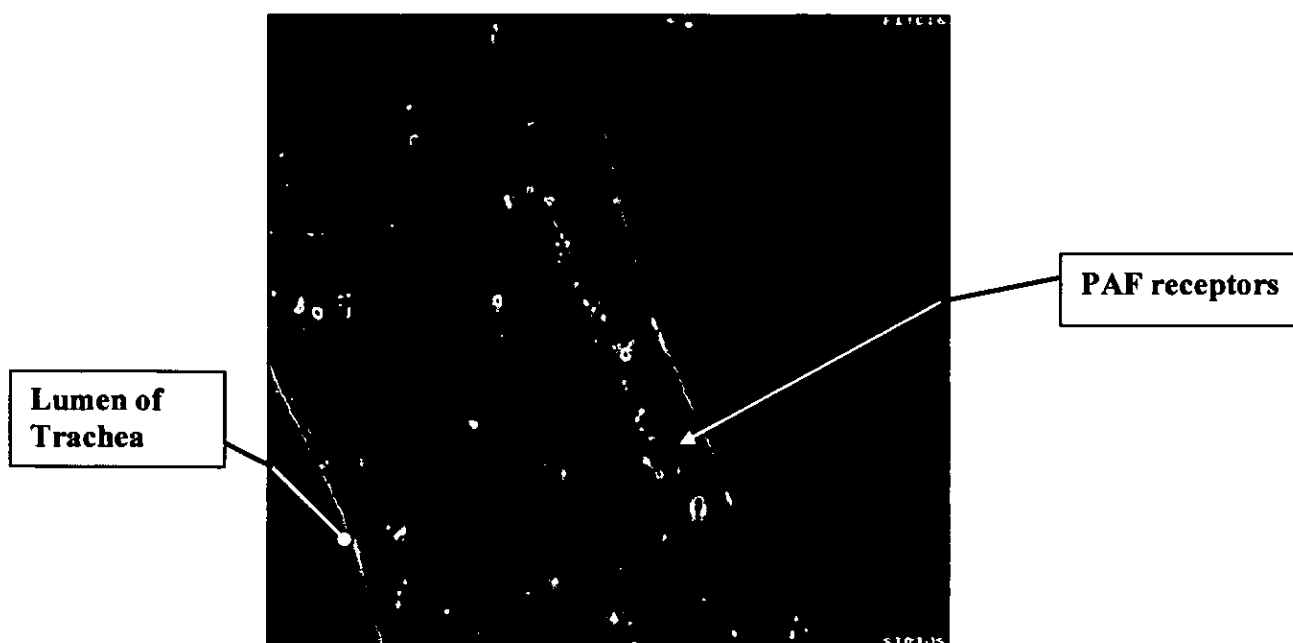
**Figure 19. Control for figure 18. Lung strip before addition of PAF, but without primary antibody.** (Magnification x 40)



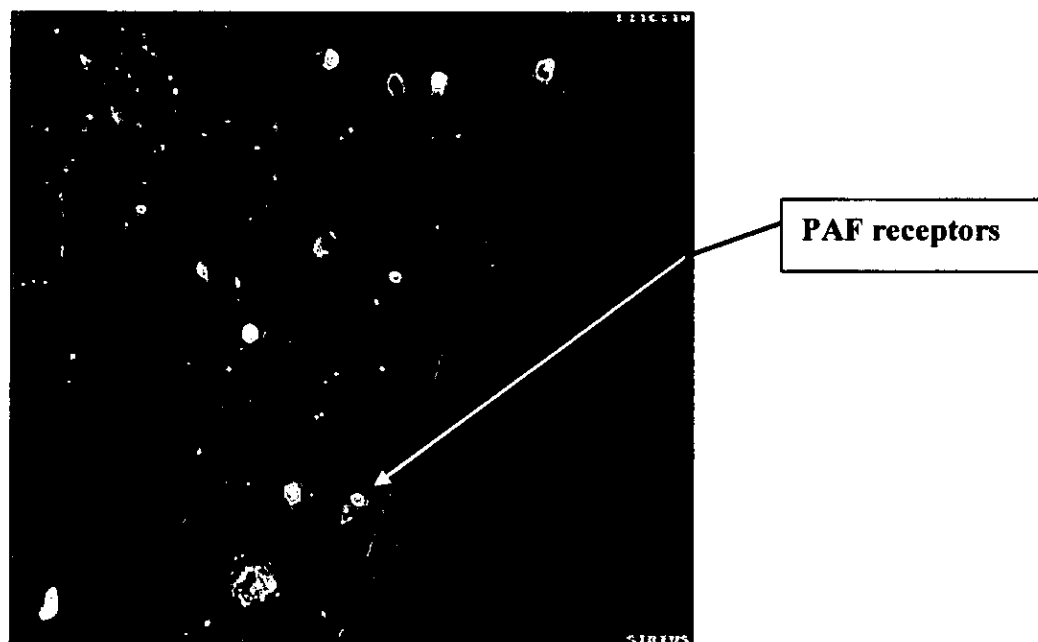
**Figure 20. PAF receptors in the lung strip after 1 exposure to PAF.  
(Magnification x 40)**



**Figure 21. PAF receptors in the lung strip after 2 exposures to PAF.  
(Magnification x 40)**



**Figure 22. PAF receptors in the trachea after one addition of PAF. (Magnification x 40)**



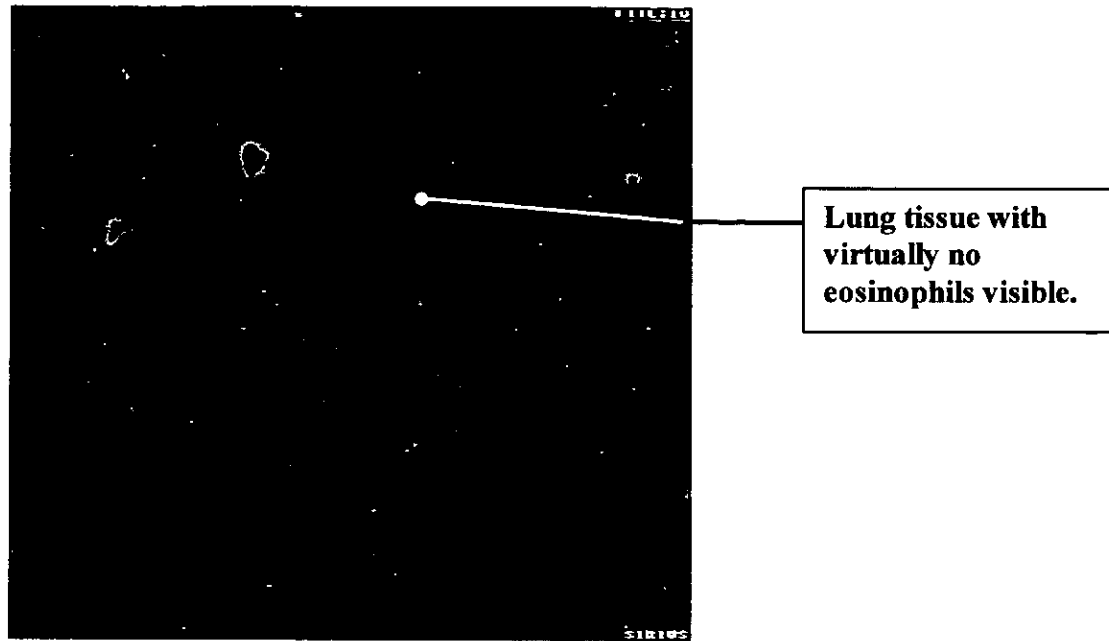
**Figure 23. PAF receptors in the trachea after two PAF exposures. (Magnification x 40)**



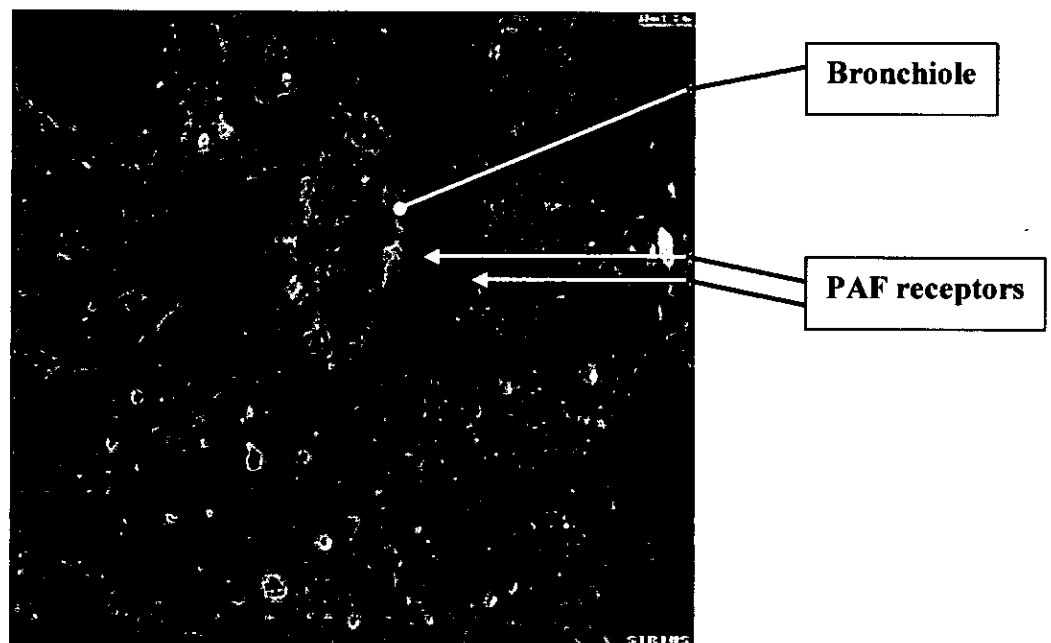
### **3.3.2 Study 4 Double Immunofluorescence (IF) of eosinophils and PAF receptors**

In figure 24, red CY3 fluorescence indicates the presence of eosinophils in the lung strip. Figures 25 and 26 show sections of the same lung strip after one and two treatments with PAF respectively. As the tissue is treated with PAF, the presence of eosinophils decreases, probably as a consequence of the washing procedure.

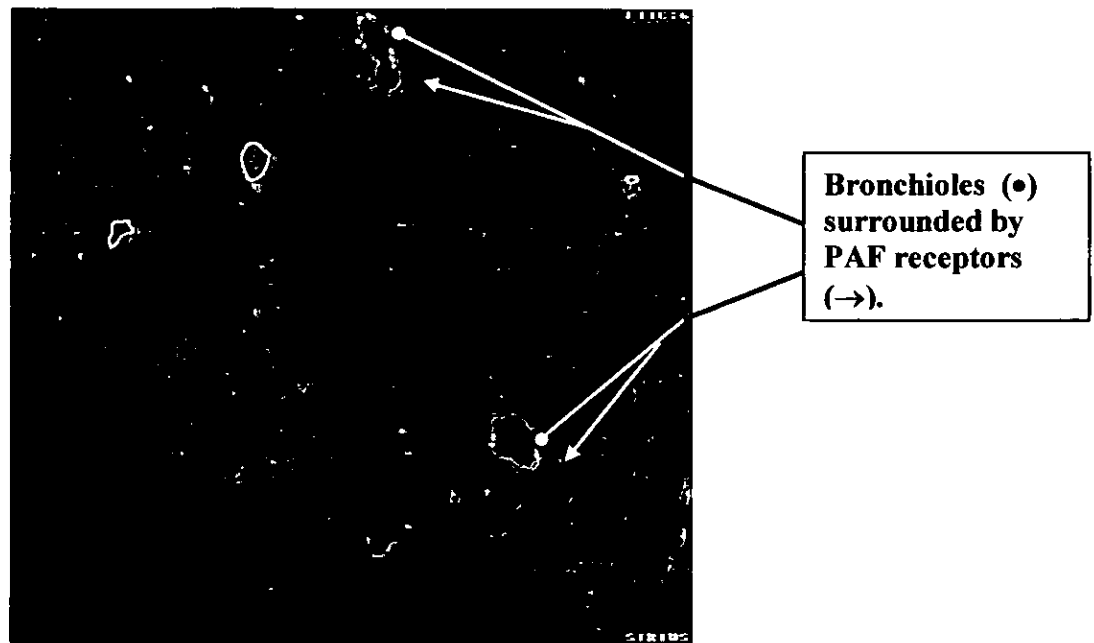
Although double IF for PAF-receptors and eosinophils was successful, it was observed that the different colours stained were best viewed individually. Figures 27 and 28 show the same sections of tissue as in figures 25 and 26 respectively, but with PAF receptor staining. In figure 27, PAF receptors occur around the bronchioles as before (see figures 20 and 21). In figure 27 PAF receptor staining is also seen elsewhere within the tissue, in contrast with figure 28 where PAF receptors are present mainly around the bronchioles. This is consistent with the hypothesis that eosinophils and their PAF receptors are gradually washed away. Similarly this is also shown in figures 20 and 21 respectively, where although PAF receptors around the bronchioles remain throughout, other staining within the tissue becomes less apparent.



**Figure 26. Eosinophils in the lung strip after 2 PAF treatments. (Magnification x 40)**



**Figure 27. PAF receptors within the lung strip after 1 exposure to PAF and double staining for eosinophils (see figure 25 for corresponding eosinophil IF). (Magnification x 40)**



**Figure 28. PAF receptor staining on the lung strip after two exposures to PAF and double staining for eosinophils (see figure 26 for corresponding eosinophil IF). (Magnification x 40)**

### **3.4 Discussion**

#### **3.4.1 Study 3 Immunofluorescent (IF) investigation into the localization of PAF receptors in the trachea and lung strip and the effect of PAF upon their localization.**

Immunofluorescence observed in this part of the study is that of an immunofluorescent tag. PAF binds to its receptor and the primary antibody, anti-PAF, then binds to this PAF. A secondary antibody with an FITC (or other such as CY3) conjugate then binds to the anti-PAF. It is the FITC (or other) conjugate which produces the immunofluorescence seen on images. If the PAF receptor were to internalise on activation by PAF, the PAF would also be internalised, leaving nowhere for anti-PAF to bind onto. This would in turn leave nowhere for the secondary antibody to bind onto, and no fluorescence would be seen.

Throughout the investigation involving immunofluorescence, simultaneous controls were produced for each slide, which in all cases produced very little immunofluorescence, indicating that any fluorescence on the test slides was specific. Immunofluorescence was used in both lung and tracheal tissue to show the presence of PAF on PAF receptors before and after PAF addition and after a subsequent PAF addition. Before the actual addition of PAF to tissue preparations, very low levels of PAF receptor staining were seen (see figure 18) which could have been the result of PAF produced by the animal itself. IF was seen after one addition of PAF and this IF was also seen after subsequent PAF additions in both lung strip and tracheal preparations. The localization of this IF was predominantly around the bronchioles of the lung tissue and along the outer edge of the trachea and did not change. The localization of PAF receptors around the bronchioles and

surrounding the trachea supports the theory of PAF being a mediator of airway hyperresponsiveness and bronchoconstriction (chapter 1). The lack of movement of PAF receptors indicates that PAF receptor internalisation, which has previously been suggested as being behind the desensitization of PAF receptors to the effects of PAF (Kloprogge and Akkerman, 1984), does not occur within airway smooth muscle. Some other mechanism must therefore be behind PAF receptor desensitization

### **3.4.2 Study 4 Double Immunofluorescence (IF) of eosinophils and PAF receptors.**

The next part of the investigation, which involved using IF to look at the possible presence of eosinophils, which could have been responsible for the large standard errors observed showed that eosinophils were present within the lung tissue (see figures 24-26). PAF receptors did appear to be present on these eosinophils, which were gradually washed away as the pharmacological procedure was followed. This part of the study once again showed that PAF receptors present within lung tissue did not move after PAF application, backing up the results found earlier in the study.

## **Chapter 4**

### **Effects of Inhibitors on PAF, 5-HT and ACh responses**

#### **4.1 Introduction**

##### **4.1.1 Study 5 Effects of d-erythro-Sphingosine (protein kinase C inhibitor) on the action of PAF *per se***

The previous chapter indicated that PAF receptors were not being internalised as the mechanism through which desensitization occurred. Thus the biochemical events following the binding of PAF to its receptor must be altered so producing desensitization.

As has been previously described, PAF can produce its action through activation of protein kinase C or PLA<sub>2</sub>. This investigation utilised enzyme inhibitors to block various points in the biochemical pathways and to determine whether PAF induced desensitization can be prevented.

Previous work (Berridge, 1987 and Nishizuka, 1986) has suggested that PAF may effect bronchoconstriction via the activation of protein kinase C (PKC) resulting from inositol 1,4,5-triphosphate (IP<sub>3</sub>) and diacylglycerol (DAG) production. DAG activates protein kinase C in the presence of calcium mobilized by IP<sub>3</sub> leading to cell activation (Henderson, 1991). It has also been suggested that PAF-induced desensitization may be a result of the activation of protein kinase C (Kamata *et al.*, 1993). It was therefore decided to investigate the effect of the PKC inhibitor sphingosine on the effects of PAF, 5-HT and ACh. Sphingosine has been shown to inhibit potently and reversibly the actions of PKC

in previous work on human platelets (Hannun and Bell, 1987). Sphingosine is a selective inhibitor of protein kinase C (Arnold and Newton, 1991).

#### **4.1.2 Study 6 Effects of MK886 (lipoxygenase inhibitor) on the action of PAF *per se***

Voelkel *et al* (1982) reported that in rat lungs, the bronchoconstriction observed following PAF addition was due to PAF inducing the release of LT's. It was the LT's which then produced the contractile response. 5-lipoxygenase is the initial enzyme in the sequence used to produce the LT's. Consequently by inhibiting this enzyme, LT's would not be produced. One of the key enzymes in the production of leukotrienes is 5-lipoxygenase activating protein (Henderson, 1991).

MK886 was reported to specifically block the actions of 5-lipoxygenase in rat lungs (Davidson and Drafton, 1992) and in human bronchi (Bjorck and Dahlen, 1993). MK886 is a 5-lipoxygenase-activating protein (FLAP) inhibitor. FLAP facilitates the transfer of AA from the membrane phospholipase to 5-lipoxygenase. Thus by using MK886 it should be possible to determine whether it is the released leukotrienes which are then inducing the desensitization of the tissue to a subsequent addition of PAF.

#### **4.1.3 Study 7 Effects of AACOCF3 (phospholipase A<sub>2</sub> inhibitor) on the action of PAF *per se***

Whilst lipoxygenase is the enzyme responsible for the production of LT's, the initial enzyme involved in LT production is the activation of PLA<sub>2</sub> leading to AA release (Chao & Olson, 1993). Thus PLA<sub>2</sub> is therefore another important site where modulation of this enzyme could lead to desensitization of the PAF response.

In order to determine the effects of PLA<sub>2</sub> activation, investigation into the effects of the PLA<sub>2</sub> inhibitor AACOCF<sub>3</sub> was proposed. Whilst there are a number of PLA<sub>2</sub> inhibitors available, AACOCF<sub>3</sub> was chosen as an inhibitor because in previous studies it has been shown to inhibit the actions of PLA<sub>2</sub> in rat isolated airway tissue (Michoud *et al.*, 1999).

#### **4.1.4 Introduction of Inhibitors to Protocol**

Determining the actions of these inhibitors was to be achieved by incubating the tissue with the appropriate inhibitor prior to the addition of PAF. If having removed the inhibitor, the tissue is then able to respond to a second addition of PAF, it would indicate that desensitization had not taken place. Thus it would be an event associated with that enzyme or the products released by it normally, which induced desensitization (see chapter 1). Alternatively, if on the removal of the inhibitor the tissue could no longer respond to PAF, it would indicate that the enzyme or agents released by the enzyme were not responsible for the desensitization process, as the desensitization had still occurred despite the presence of the inhibitor.

In order to ensure this study was comparable with the original experiments using PAF (chapter 2) a similar protocol was used as previously. Following the initial ACh curves in the absence and presence of 5-HT the inhibitor was equilibrated prior to the addition of PAF. Once the tissue had been extensively washed to remove the inhibitor, the effect of PAF, 5-HT and ACh were again examined.



Whilst it was known that constrictor actions on the lung are due to LT release (Voelkel *et al.*, 1982). It was however, possible that other actions of PAF on lung tissue did not act through LT release, or were even being masked by the LT mediated effects. Hence the use of 5-HT and ACh in the presence of the inhibitors may reveal altered responsiveness of the tissue.

## 4.2 Methods

### 4.2.1 Study 5 Effects of Sphingosine (protein kinase C inhibitor) on the action of PAF *per se*

Four ACh concentration effect curves were performed on each individual tissue preparation of trachea or lung strip. Initially two ACh concentration effect curves were performed (as in chapter 2.2), one curve with ACh alone and the second after application of either 5-HT or ascorbic acid. Two further curves were performed after the following treatments:

Once the tissue had returned to its original baseline sphingosine was added to each tissue preparation, at a final concentration of 20 $\mu$ M and with a contact time of 30 min. Ethanol was used as a solvent matched control against the sphingosine.

The effects of single doses of 1 $\mu$ M PAF were determined for each preparation using contact times of 3 min. The effects of 30 $\mu$ M 5-HT were determined as before with ascorbic acid used as a control, and a contact time of 15 min. Subsequently, ACh concentration effect curves were performed.

In order to ensure that the inhibitor was properly washed from the tissue, each organ bath was irrigated by overflow with 1L of BSA free Greenberg-Bohr buffer, before the baths were re-filled with GBB-BSA. Tissues were allowed to return to baseline and left to equilibrate for 30 min.

PAF and either 5-HT or ascorbic acid were added to the preparations as before, in similar concentrations and contact times. ACh concentration effect curves were repeated, and tissue wet weights recorded.

#### **4.2.2 Study 6 Effects of MK886 (lipoxygenase inhibitor) on the action of PAF *per se***

Study 5 was repeated, replacing sphingosine (20 $\mu$ M) with MK886 (20 $\mu$ M).

#### **4.2.3 Study 7 Effects of AACOCF3 (protein kinase C inhibitor) on the action of PAF *per se***

Study 5 was repeated, replacing sphingosine (20 $\mu$ M) with AACOCF3 (10 $\mu$ M).

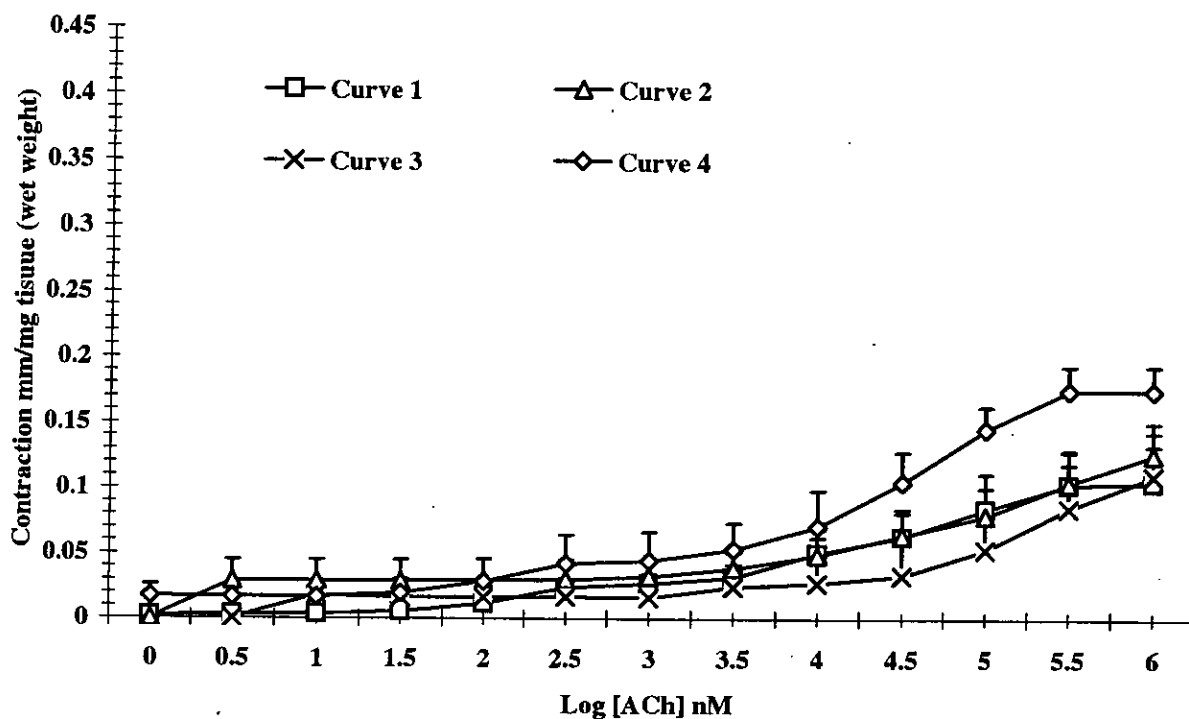
## 4.3 Results

### 4.3.1 Study 5 Effects of Sphingosine (protein kinase C inhibitor) on the action of ACh, 5-HT and PAF *per se*

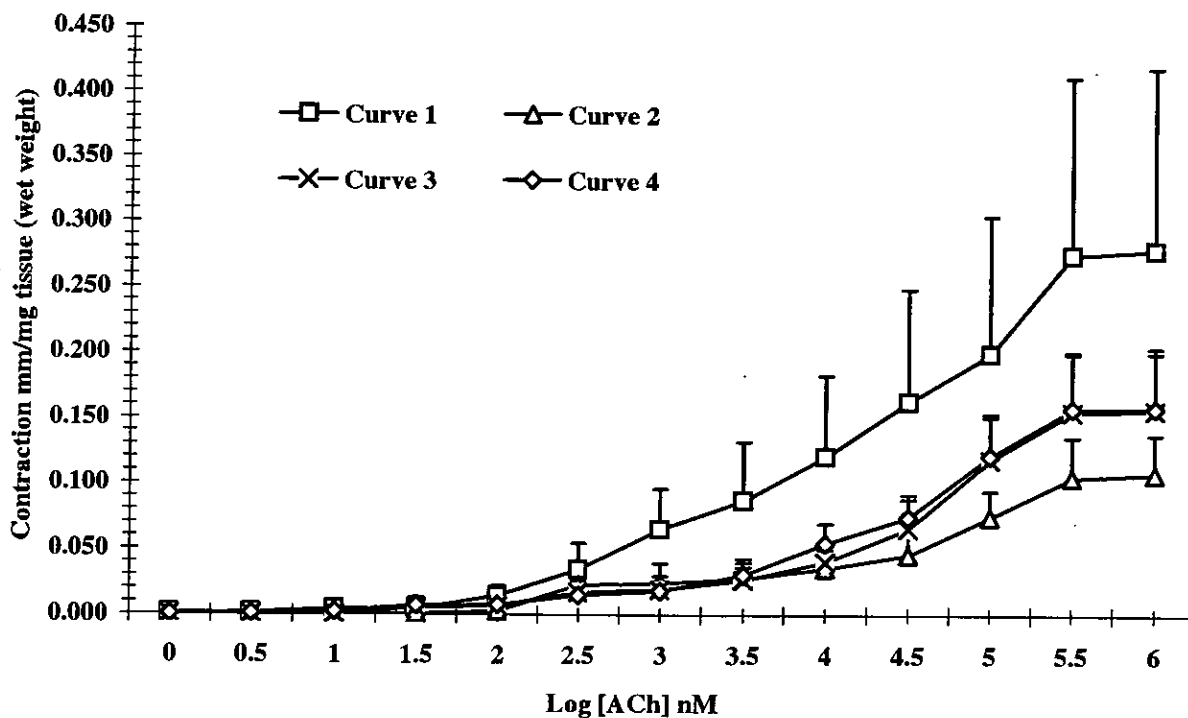
There was no significant difference between the responses to ACh in either tracheal spirals or lung strips in the presence of sphingosine compared to their controls (figures 29-32).

Tracheal contractions following addition of 5-HT alone, 5-HT in the presence of sphingosine and PAF, and then 5-HT in the presence of a second PAF addition were not significantly different from their controls (figure, 33). Similar results were obtained for the lung strip (figure, 35).

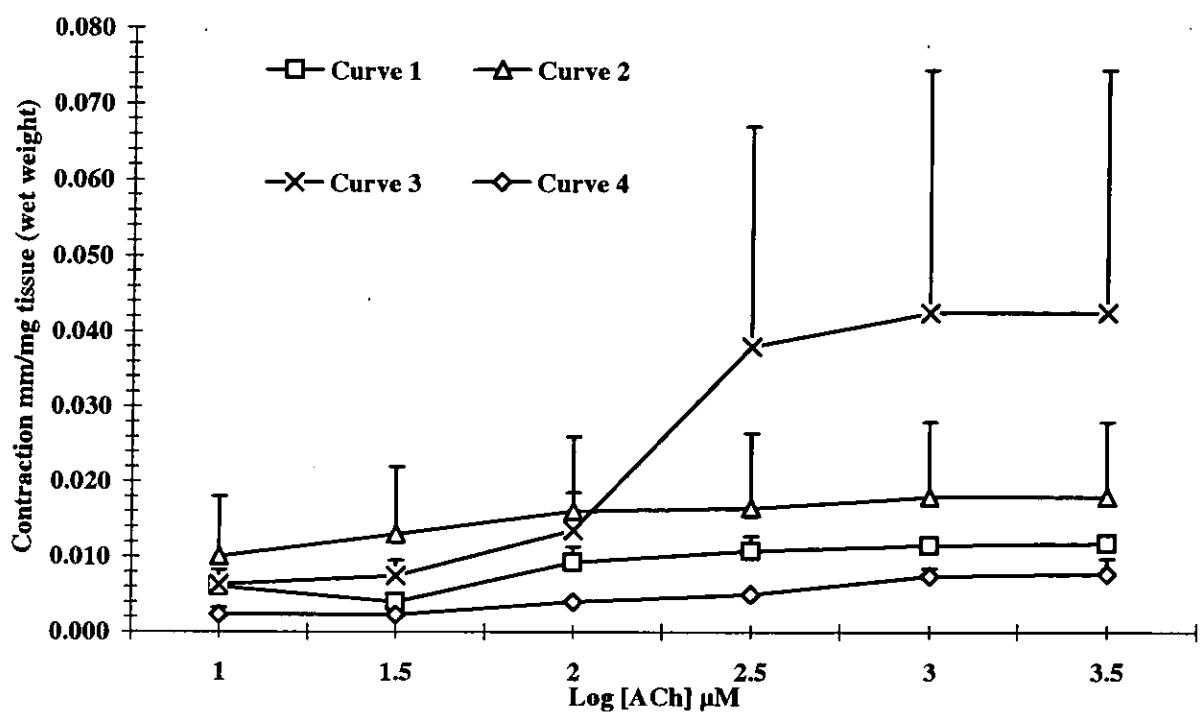
Following addition of PAF in the presence of sphingosine, both tracheal spirals and lung strips contracted (figures 34 and 36 respectively). Following a second addition of PAF, very little or no contraction was observed from either tissue. In the case of the trachea, contractions produced after a second addition of PAF were significantly lower than those observed after the first addition ( $p = 0.022$ , ANOVA). Similar observations were observed in the lung strip, although the differences between responses did not reach significance. No tissue responses to PAF in the presence of sphingosine were significantly different from their controls. Worth noting however, in the control lung strip preparation which had received no inhibitor, the initial response to PAF was significantly higher than the response to subsequent PAF addition ( $p = 0.001$ ).



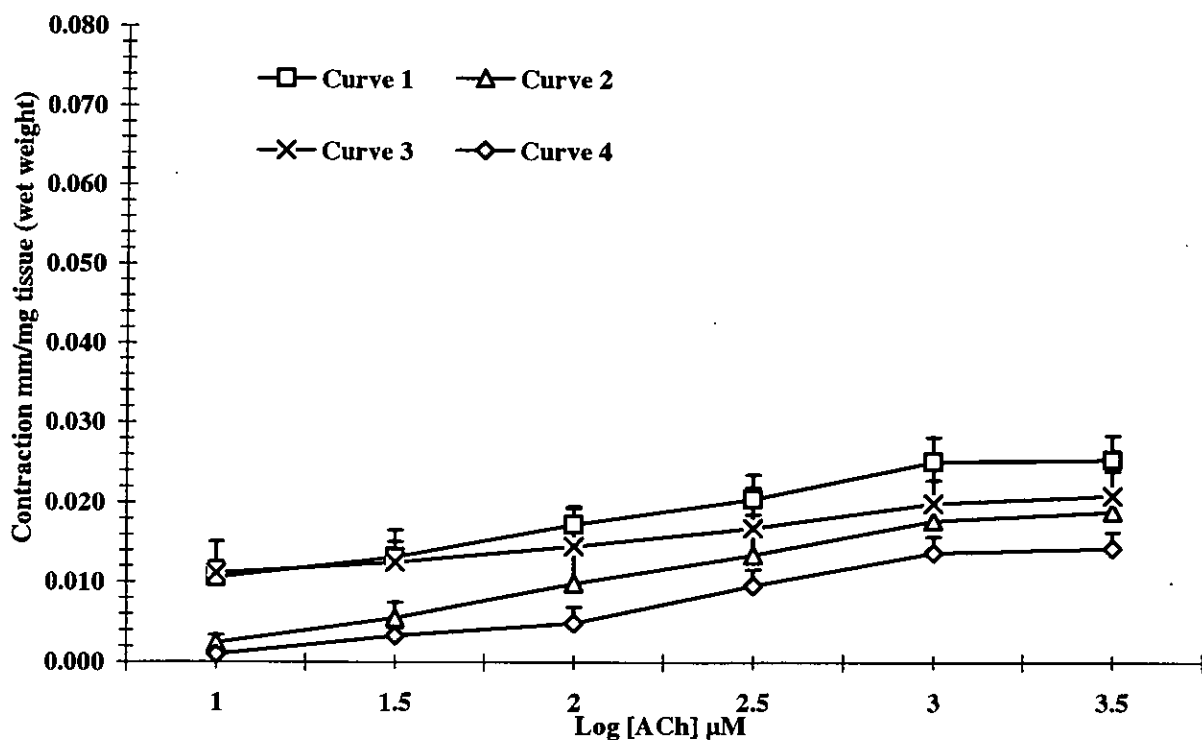
**Fig. 29 Repeated ACh concentration effect curves on rat isolated tracheal spirals.**  
 Curve 1, ACh alone; Curve 2, with 5-HT; Curve 3, with Sphingosine, PAF and 5-HT;  
 Curve 4, with PAF and 5-HT. Values expressed as mean  $\pm$  S. E. M., n=4



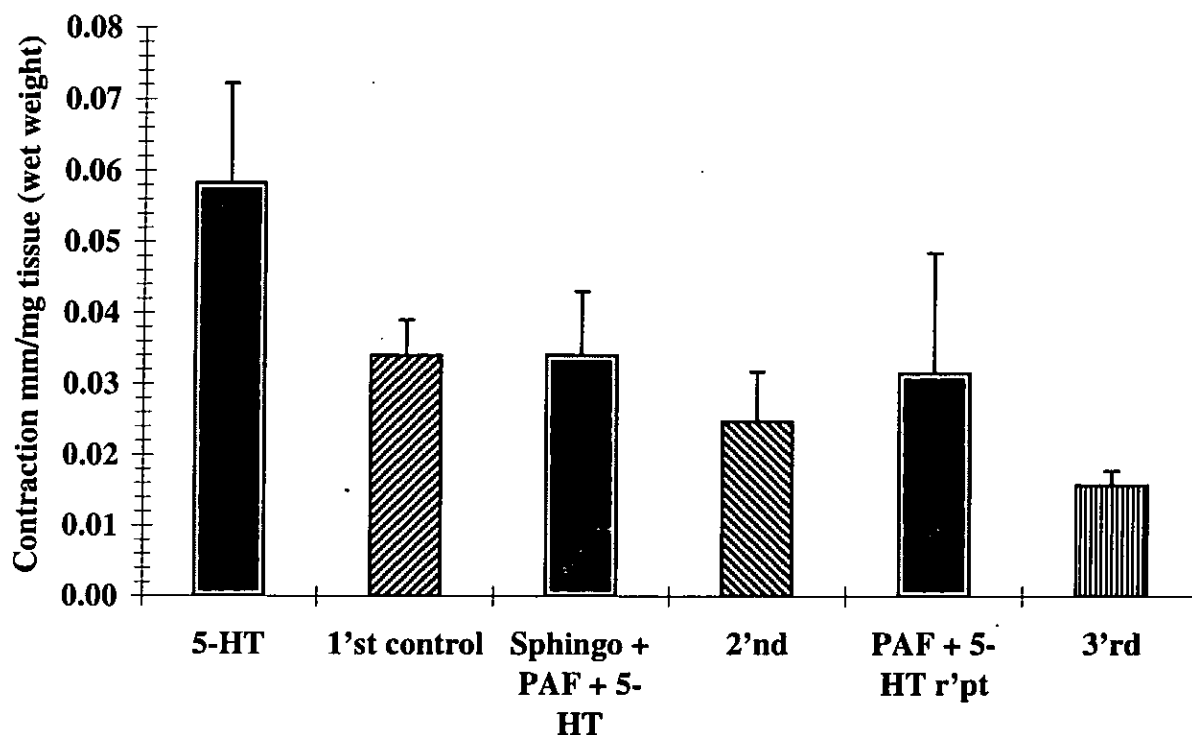
**Fig. 30 Repeated ACh concentration effect curves on rat isolated tracheal spirals**  
 Curve 1, ACh alone; Curve 2, with ascorbic acid (aa); Curve 3, Ethanol, PAF and aa;  
 Curve 4, PAF and aa. Values expressed as mean  $\pm$  S. E. M., n=8.



**Fig. 31 Repeated ACh concentration effect curves on rat isolated lung strips.**  
 Curve 1, ACh alone; Curve 2, with 5-HT; Curve 3, with Sphingosine, PAF and 5-HT;  
 Curve 4, with PAF and 5-HT. Values expressed as mean  $\pm$  S. E. M.,  $n=4$

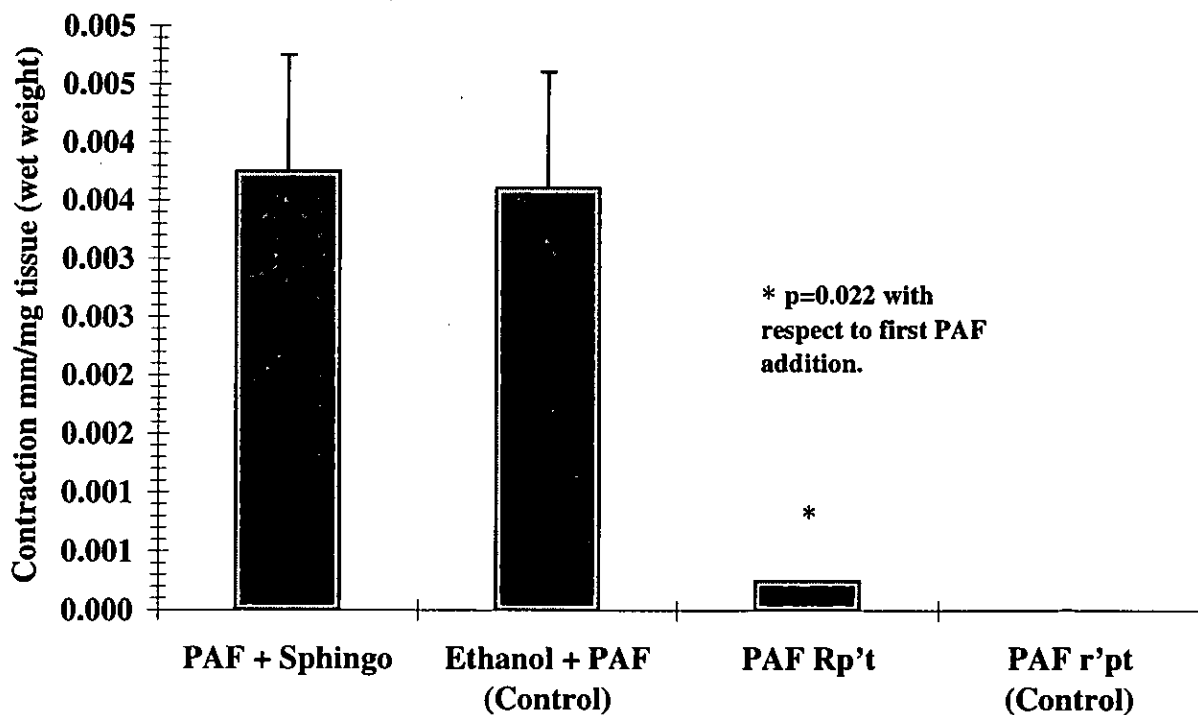


**Fig. 32 Repeated ACh concentration effect curves on rat isolated lung strips**  
 Curve 1, ACh alone; Curve 2, with ascorbic acid (aa); Curve 3, Ethanol, PAF and aa;  
 Curve 4, PAF and aa. Values expressed as mean  $\pm$  S. E. M.,  $n=8$ .



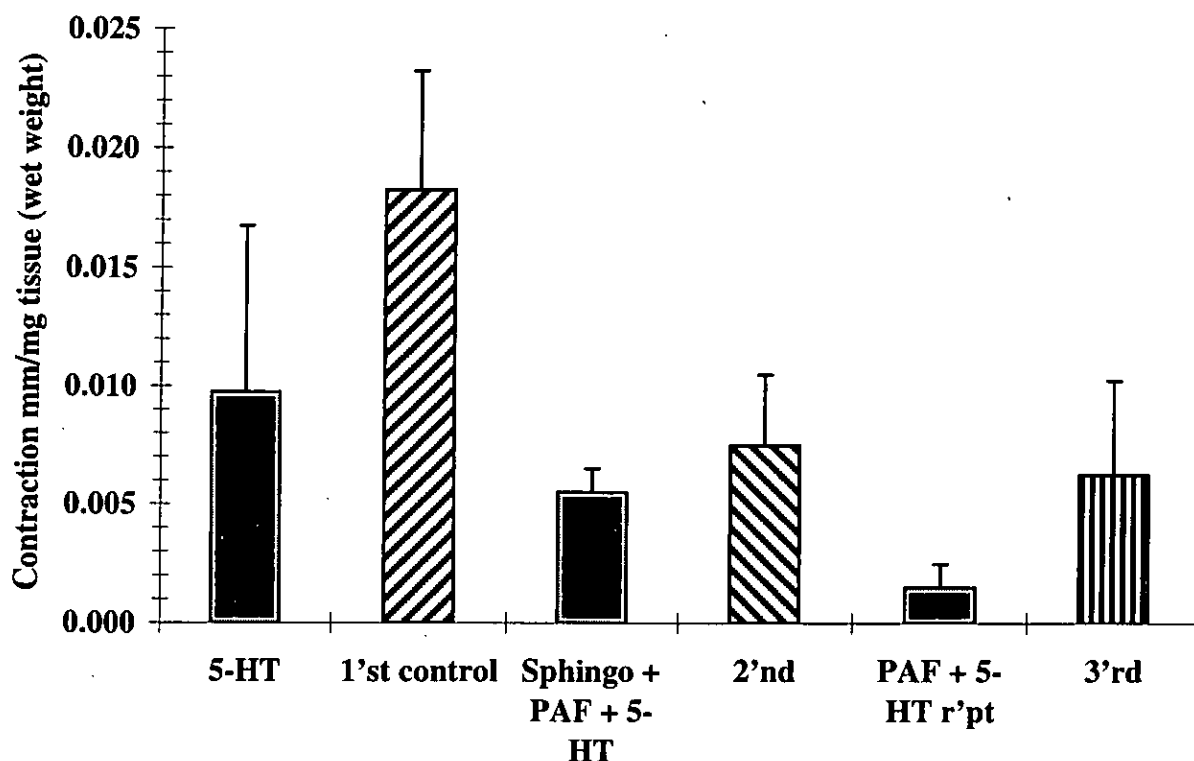
**Fig. 33 Responses given by tracheal spirals on addition of 5-HT in the presence and absence of PAF and the PKC inhibitor, sphingosine.**

Values expressed as mean  $\pm$  S. E. M., n=4.

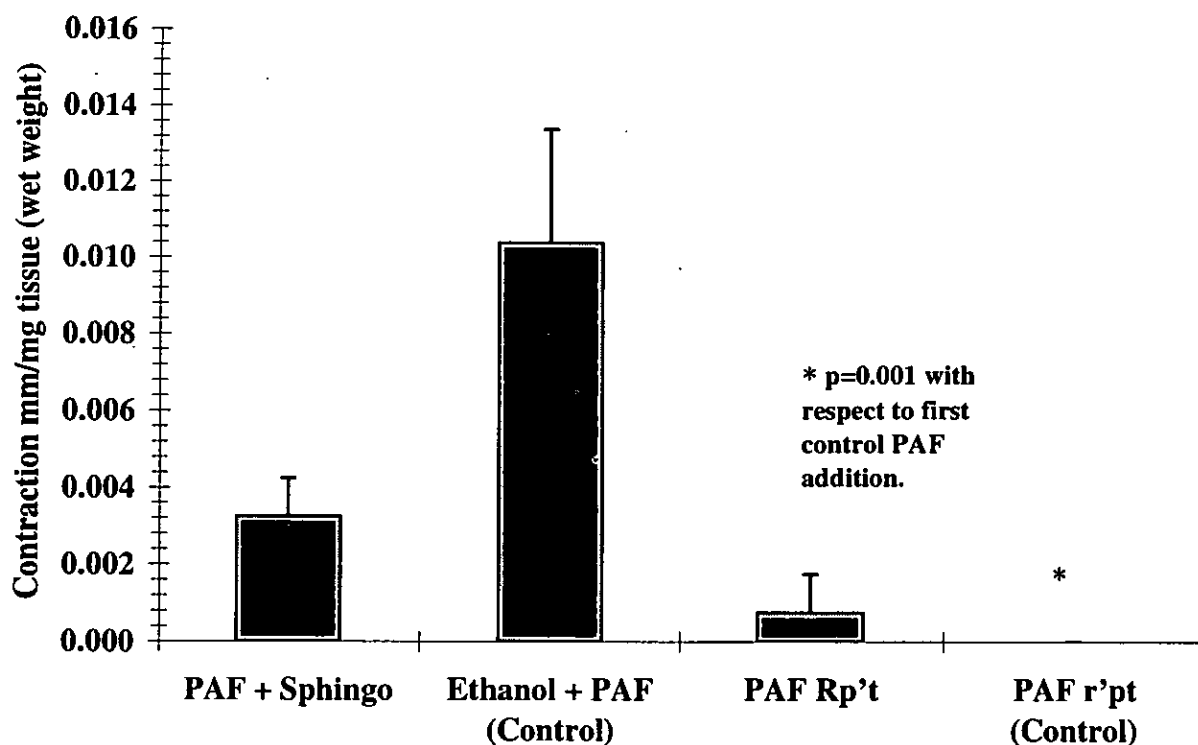


**Fig. 34 Responses given by tracheal spirals on repeated additions of PAF in the presence and absence of the PKC inhibitor, sphingosine.**

Values expressed as mean  $\pm$  S. E. M., n=4, and n=8 for controls.



**Fig. 35 Responses given by lung strips on addition of 5-HT in the presence and absence of PAF and the PKC inhibitor, sphingosine.**  
 Values expressed as mean  $\pm$  S. E. M., n=4.



**Fig. 36 Responses given by lung strips on repeated additions of PAF in the presence and absence of the PKC inhibitor, sphingosine.**  
 Values expressed as mean  $\pm$  S. E. M., n=4, and n=8 for controls.

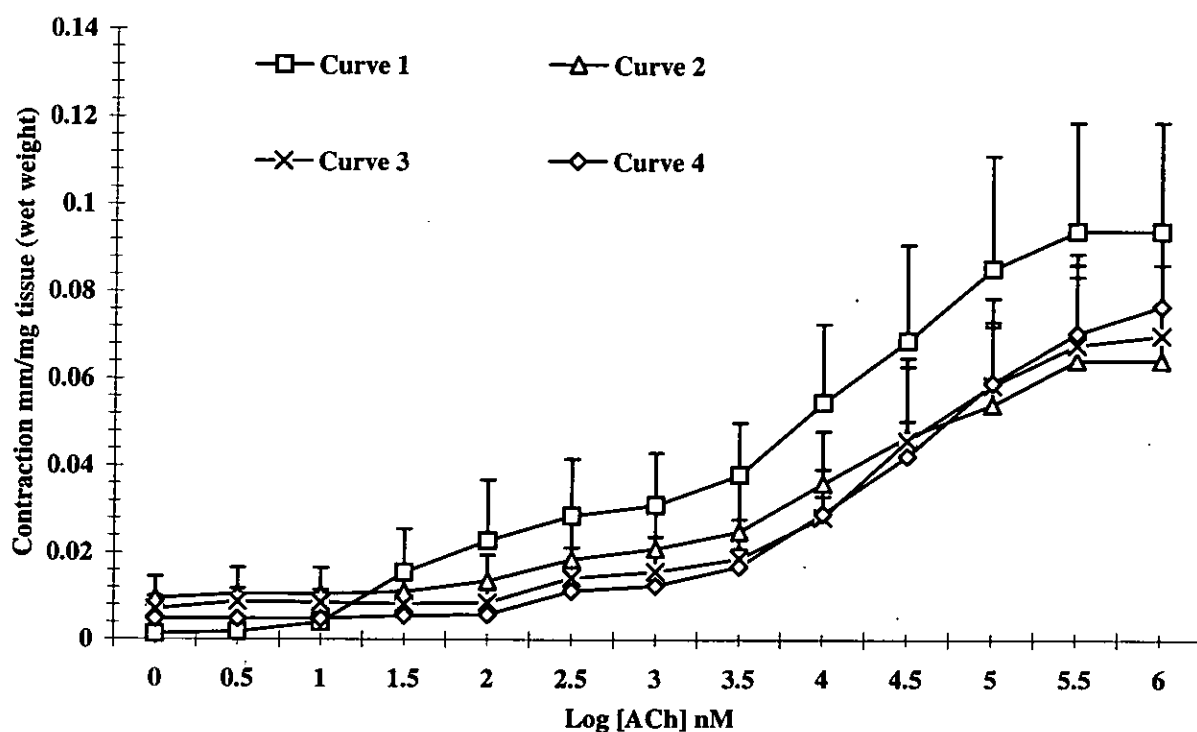


#### **4.3.2 Study 6 Effects of MK886 (lipoxygenase inhibitor) on the action of ACh, 5-HT and PAF *per se***

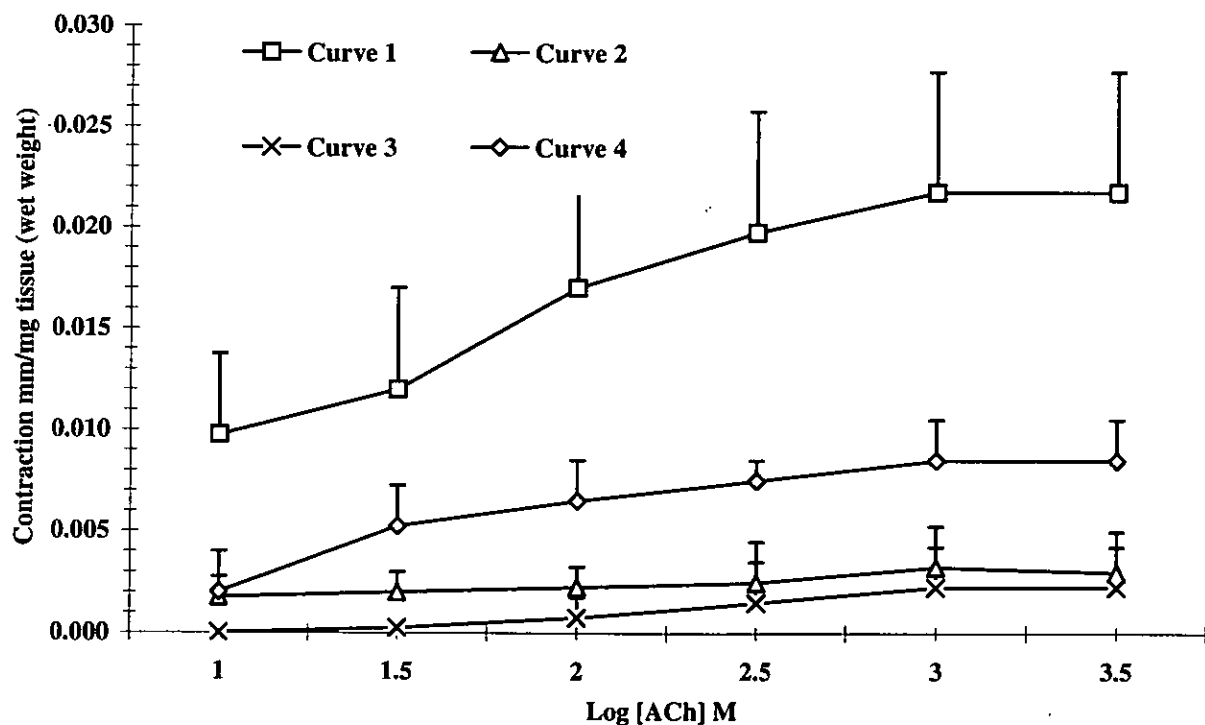
As with sphingosine, no statistically significant differences were found between ACh concentration effect curves and their controls in either the tracheal (see figure 37; 30 for control) or lung strip preparations (see figure 38; figure 32 for control).

In the cases of both the trachea and the lung strip, the contractions given on addition of 5-HT were reduced with repetition, and were not significantly different from their controls (see fig. 39 and 41 respectively) where no MK886 had been added.

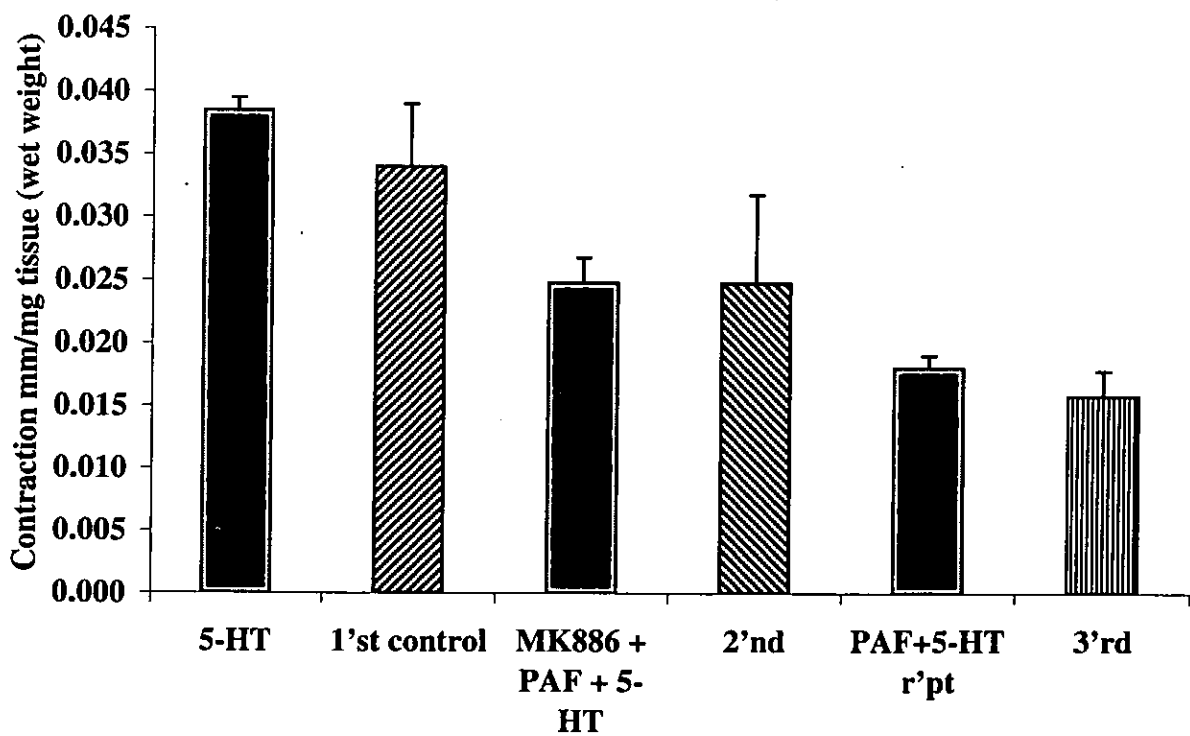
Tracheal preparations, in the presence of MK886, gave no response on addition of PAF. Although the tissue was washed to remove MK886, a second addition of PAF failed to induce a contraction (see fig. 40). Similar results were obtained in the lung strip, although extremely small responses to PAF occurred on both additions (see fig. 42), which did not reach statistical significance. It is probable that these were the result of tissues not properly returning to baseline before PAF addition.



**Fig. 37 Repeated ACh concentration effect curves on tracheal spirals**  
 Curve 1, ACh alone; Curve 2, with 5-HT; Curve 3, with MK886, PAF and 5-HT; Curve 4, with PAF and 5-HT. Values expressed as mean  $\pm$  S. E. M., n=4

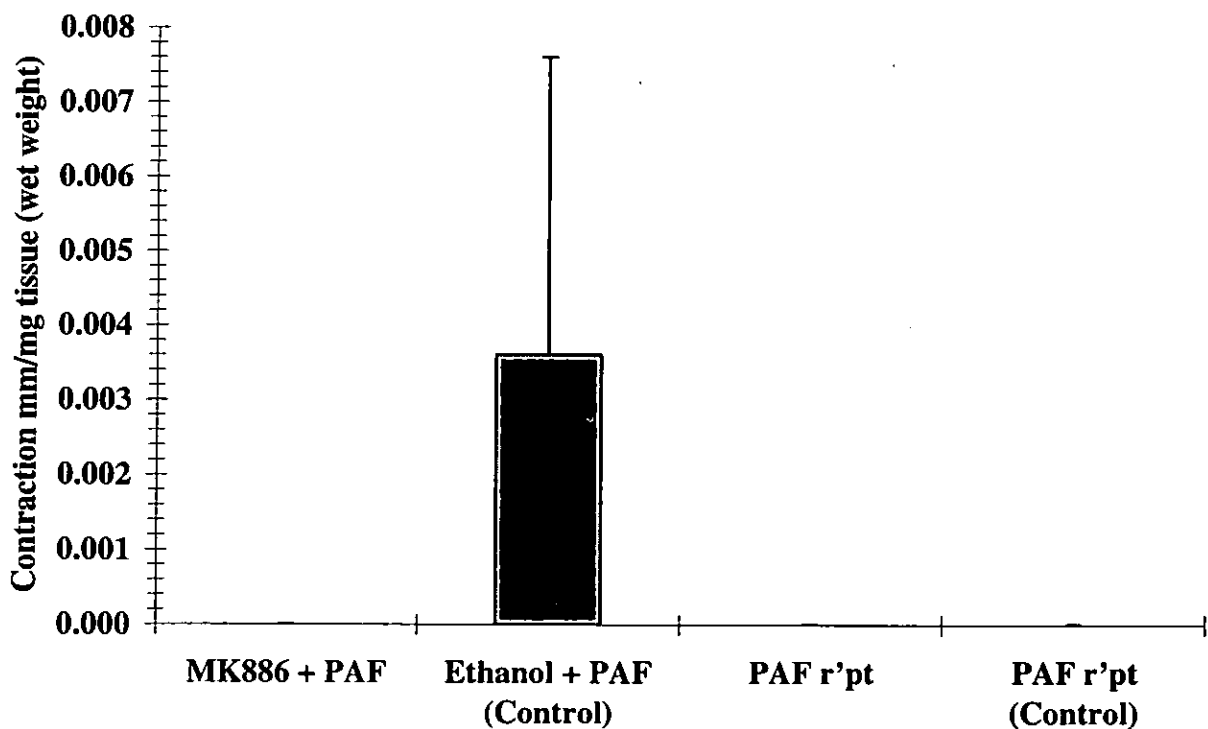


**Fig. 38 Repeated ACh concentration effect curves on rat isolated lung strips**  
 Curve 1, ACh alone; Curve 2, with 5-HT; Curve 3, with MK886, PAF and 5-HT; Curve 4, with PAF and 5-HT. Values expressed as mean  $\pm$  S. E. M., n=4



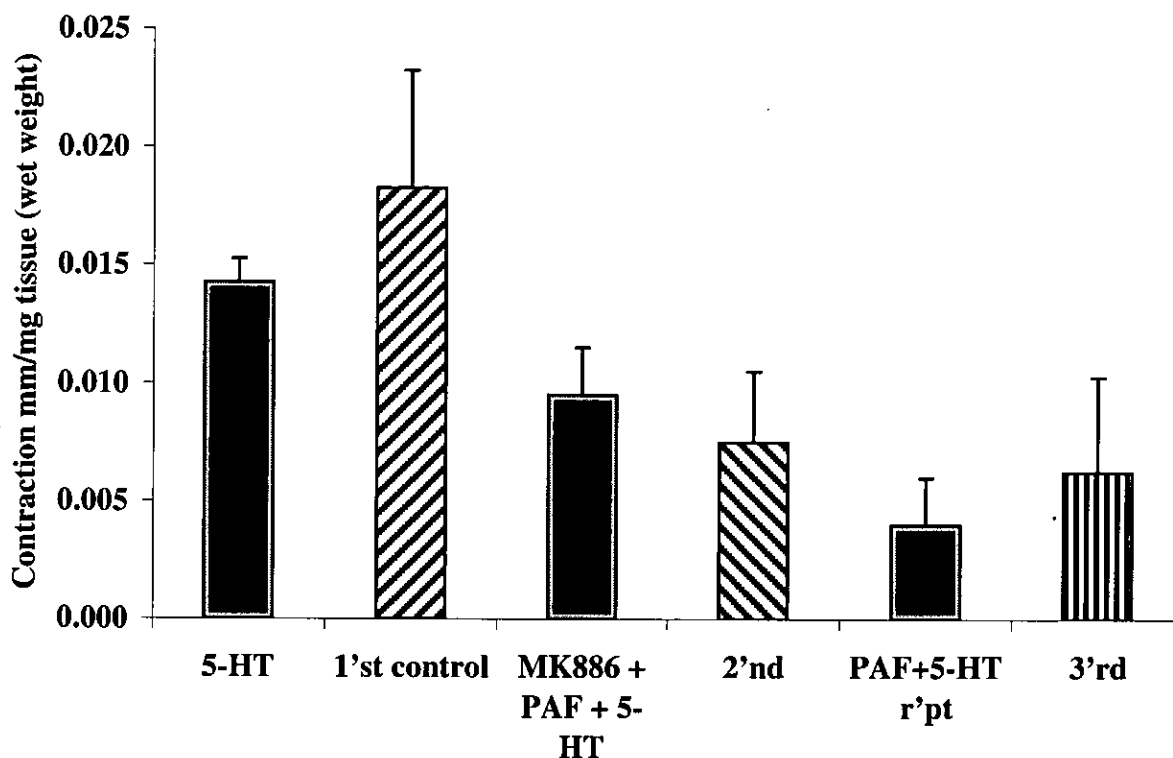
**Fig. 39 Responses given by tracheal spirals on addition of 5-HT in the presence and absence of PAF and the lipoxxygenase inhibitor MK886.**

Values expressed as mean  $\pm$  S. E. M., n=4.

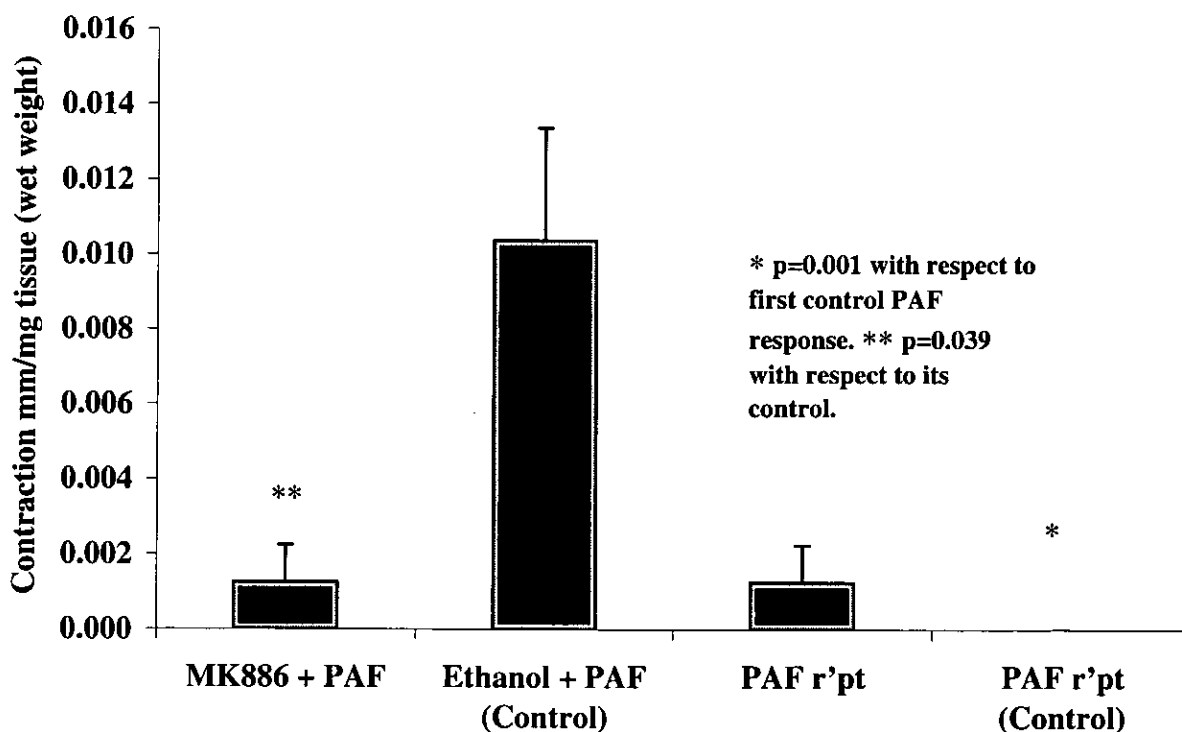


**Fig. 40 Responses given by tracheal spirals on repeated additions of PAF in the presence and absence of the lipoxxygenase inhibitor MK886.**

Values expressed as mean  $\pm$  S. E. M., n=4, and n=8 for controls.



**Fig. 41 Responses given by lung strips on addition of 5-HT in the presence and absence of PAF and the lipoxigenase inhibitor MK886.**  
Values expressed as mean  $\pm$  S. E. M., n=4.



**Fig. 42. Responses given by tracheal spirals on repeated additions of PAF in the presence and absence of the lipoxigenase inhibitor MK886.**

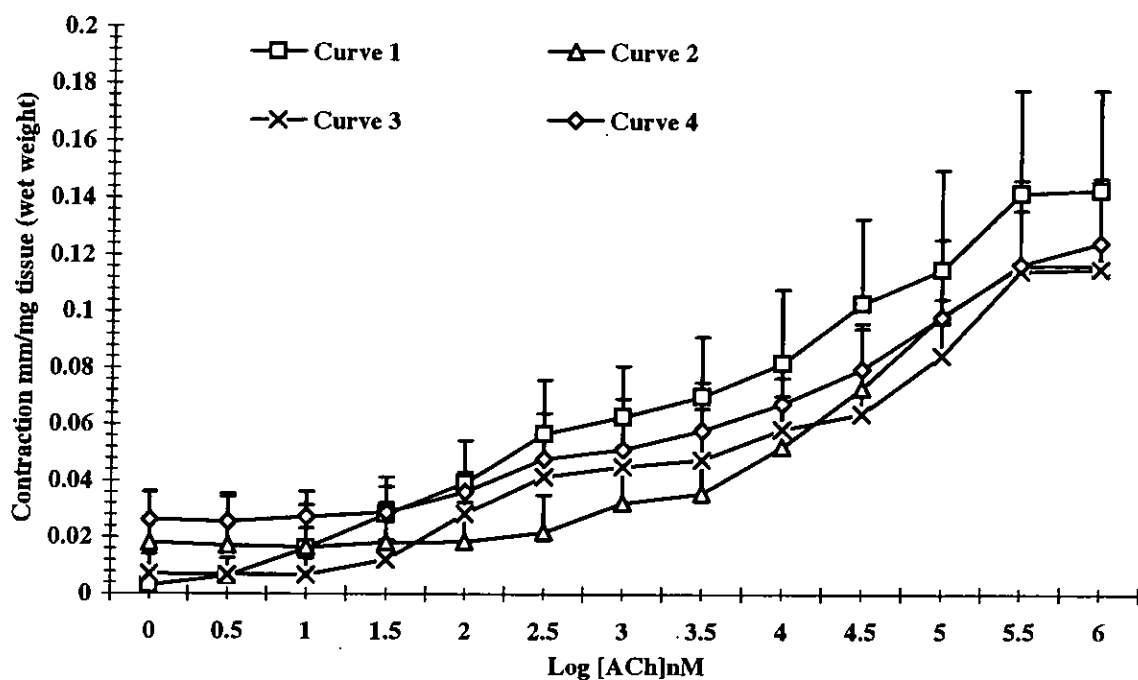
Values expressed as mean  $\pm$  S. E. M., n=4, and n=8 for controls.

#### **4.3.3 Study 7 Effects of AACOCF3 (phospholipase A<sub>2</sub> inhibitor) on the action of ACh, 5-HT and PAF *per se***

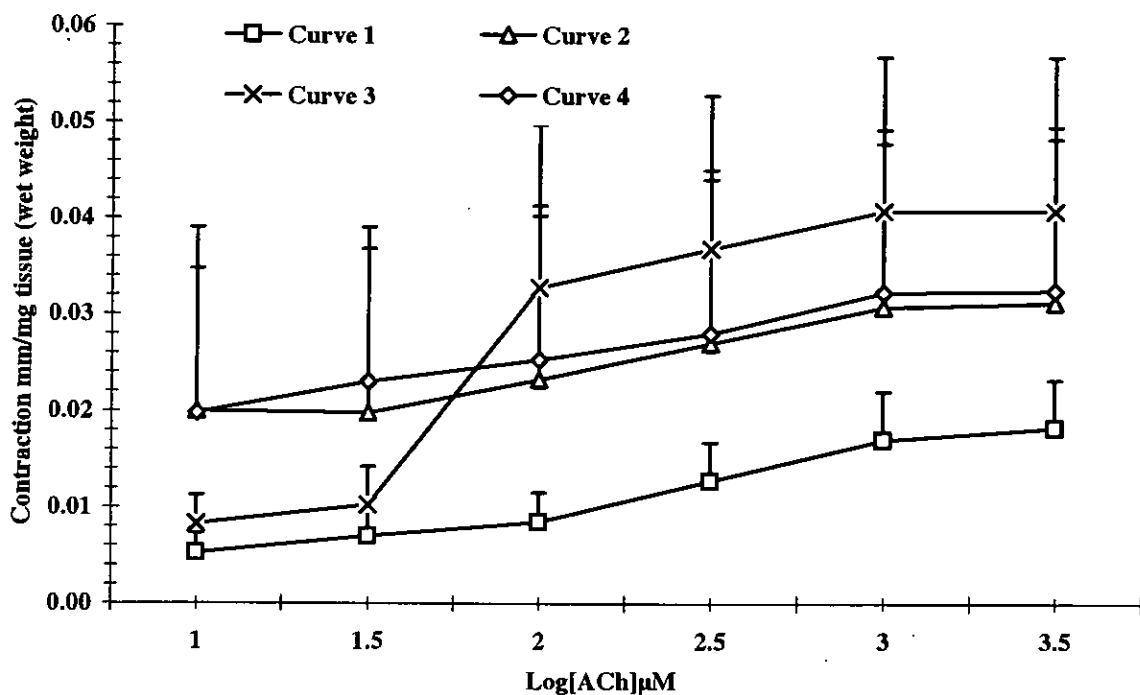
No statistically significant differences were observed between the ACh concentration effect curves in the presence of AACOCF3 and their controls in either the tracheal (see figure 43; 30 for control) or lung strip preparations (see figure 44; 32 for control).

In both tissue types, contractions given on addition of a second dose of 5-HT in the presence of AACOCF3 were smaller than those obtained after the initial 5-HT additions (see figures 45 – trachea, and 47 – lung strip). The first and second 5-HT responses showed no significant differences between their controls. On a third addition of 5-HT however, once AACOCF3 had been washed away, both lung strip and tracheal preparations gave contractions greater in size than the contractions produced after the second 5-HT addition. In both tissue types these contractions were greater than those given by their controls although this only reached statistical significance in the tracheal preparations ( $p = 0.018$ ).

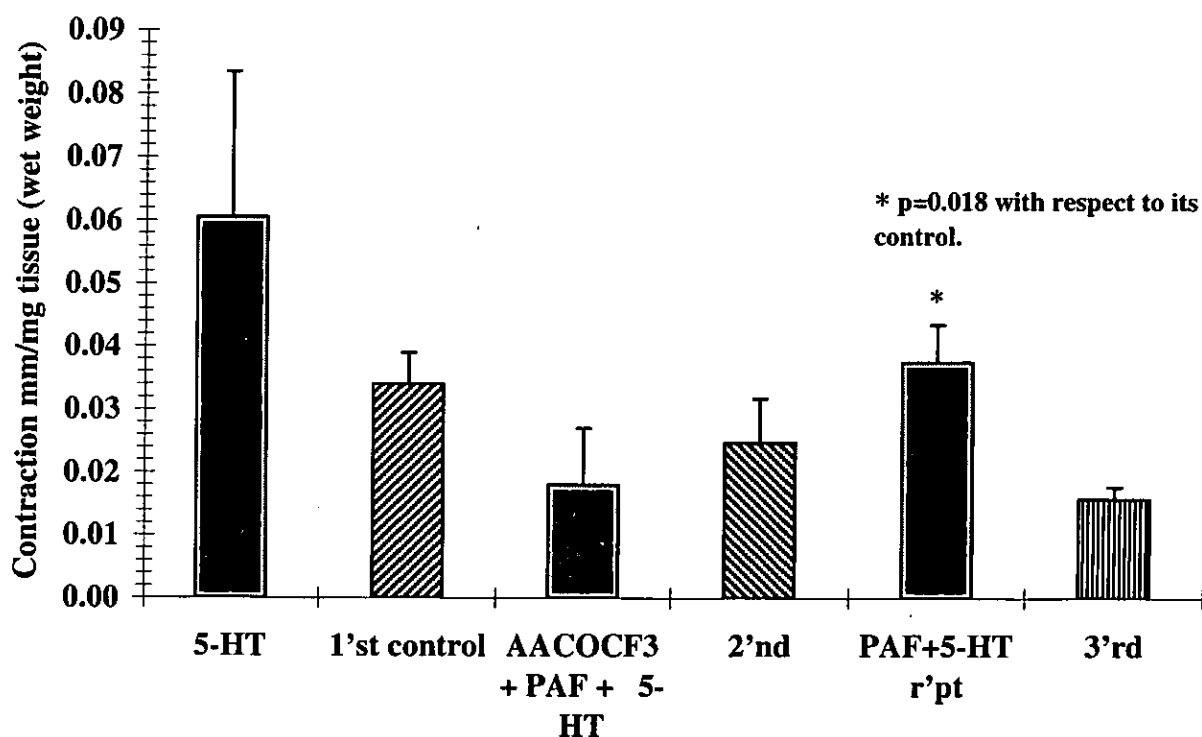
Addition of PAF in the presence of AACOCF3 affected no responses in either tracheal or lung strip preparations (see figures 46 and 48 respectively). Tissue contractions were observed in the trachea and lung strip after removal of AACOCF3 and a second PAF application. In both the trachea and lung strip preparations the second PAF responses were significantly higher than their controls ( $p = 0$  and  $p = 0.003$  respectively). For tracheal preparations, differences between the first and second PAF responses also reached significance ( $p = 0.01$ ).



**Fig. 43 Repeated ACh concentration effect curves on rat isolated tracheal spirals.**  
 Curve 1, ACh alone; Curve 2, with 5-HT; Curve 3, with AACOCF3, PAF and 5-HT;  
 Curve 4, with PAF and 5-HT. Values expressed as mean  $\pm$  S. E. M., n=4

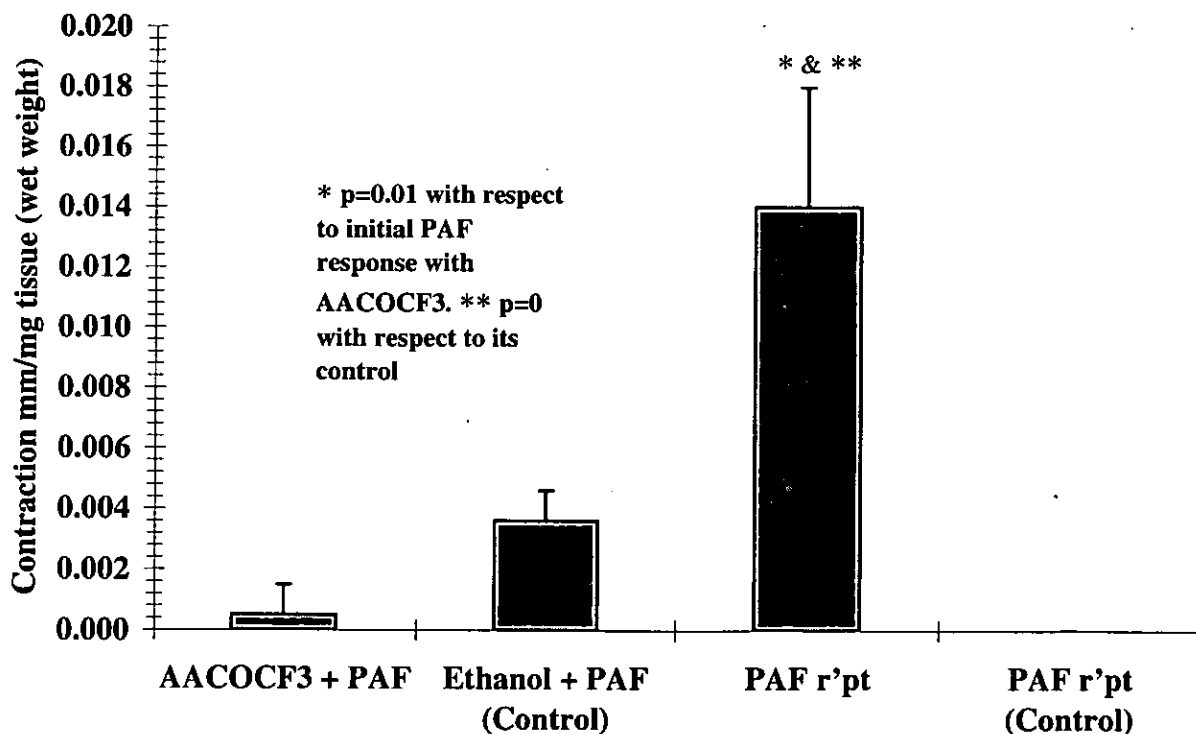


**Fig. 44 Repeated ACh concentration effect curves on rat isolated lung strips.**  
 Curve 1, ACh alone; Curve 2, with 5-HT; Curve 3, with AACOCF3, PAF and 5-HT;  
 Curve 4, with PAF and 5-HT. Values expressed as mean  $\pm$  S. E. M., n=4



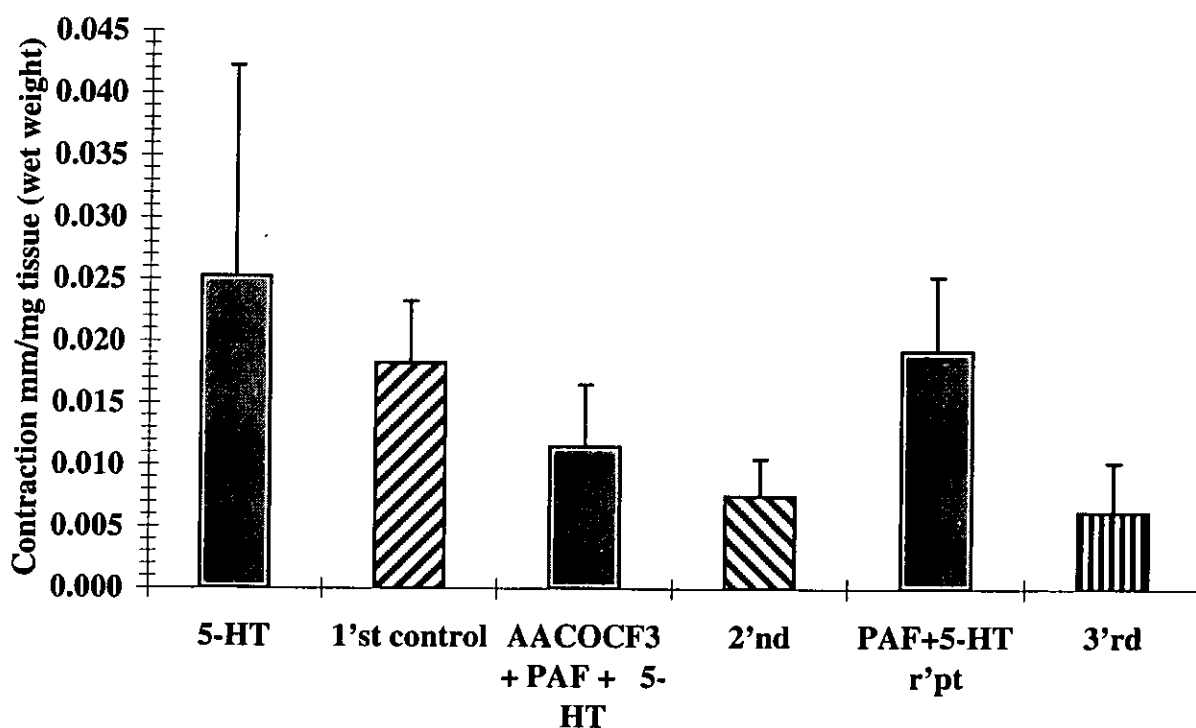
**Fig. 45 Responses given by tracheal spirals on addition of 5-HT in the presence and absence of PAF and the phospholipase A2 inhibitor AACOCF3.**

Values expressed as mean  $\pm$  S. E. M., n=4.



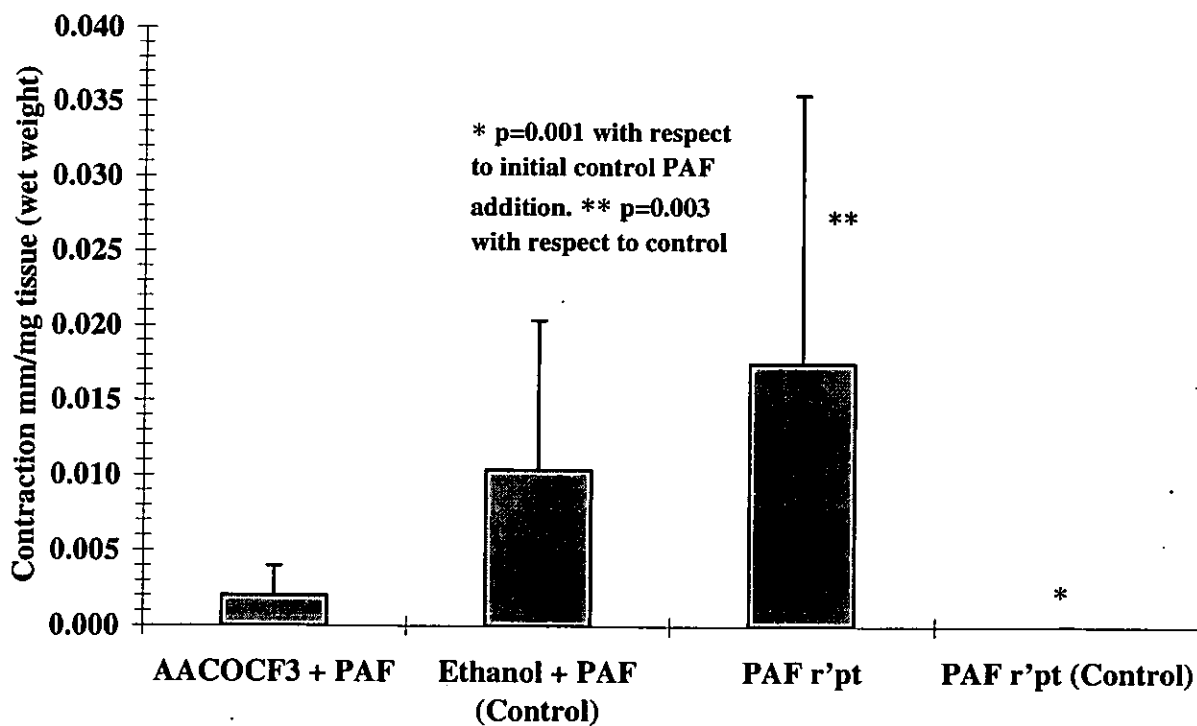
**Fig. 46 Responses given by tracheal spirals on repeated additions of PAF in the presence and absence of the phospholipase A2 inhibitor AACOCF3.**

Values expressed as mean  $\pm$  S. E. M., n=4, and n=8 for controls.



**Fig. 47 Responses given by lung strips on addition of 5-HT in the presence and absence of PAF and the phospholipase A2 inhibitor AACOCF3.**

Values expressed as mean  $\pm$  S. E. M., n=4.



**Fig. 48 Responses given by lung strips on repeated additions of PAF in the presence and absence of the phospholipase A2 inhibitor AACOCF3.**

Values expressed as mean  $\pm$  S. E. M., n=4, and n=8 for controls.



## **4.4 Discussion**

### **4.4.1 Study 5 Effects of Sphingosine (protein kinase C inhibitor) on the action of PAF *per se***

Inhibiting the enzyme protein kinase C had no effect upon the actions of ACh or 5-HT in either the presence or absence of PAF. Contractions given by lung strips and tracheal spirals in response to repeated PAF application were also not affected by the inhibition of protein kinase C, and desensitization was observed as before.

This could indicate that the actions of PAF are not the result of a protein kinase C mediated pathway. This is supported by work with rabbit platelets (Shimizu *et al.*, 1992) also indicating that a protein kinase C mediated pathway is not involved in the desensitization of receptors to the actions of PAF. Alternatively the concentration of sphingosine chosen for use (20 $\mu$ M) was not sufficient to inhibit protein kinase C activation. Work by Hannun and Bell (1987) however showed that concentrations of between 5 $\mu$ M and 20 $\mu$ M were sufficient to inhibit the actions of protein kinase C.

### **4.4.2 Study 6 Effects of MK886 (lipoxygenase inhibitor) on the action of PAF**

Inhibition of lipoxygenase had no effects upon tissue contractions in response to either ACh or 5-HT.

The initial response to PAF was however not observed in either tissue type when lipoxygenase was inhibited. Subsequent PAF application also produced no PAF response, so desensitization of PAF receptors to PAF had still occurred. This showed that PAF does

act via leukotrienes, confirming the findings of Voelkel *et al* (1982) and of Warhurst and Court (1996). This also indicated that the production of leukotrienes could not be responsible for the desensitization of PAF receptors to the effects of PAF, therefore suggesting that desensitization occurred as the result of earlier events in the biochemical pathway. Alternatively it could suggest that MK886 had not been properly washed from tissues prior to subsequent PAF addition. The work of Davidson and Drafton (1992) also using rat lungs however suggested that MK886 could be removed from the tissue and that the inhibitor was not irreversible.

#### **4.4.3 Study 7 Effects of AACOCF<sub>3</sub> (protein kinase C inhibitor) on the action of PAF**

Inhibition of phospholipase A<sub>2</sub> had no effect upon the contractile responses of tissues in response to either ACh or 5-HT. This indicated that neither ACh or 5-HT were acting through a PLA<sub>2</sub> mediated pathway.

The contractile effects of an initial PAF addition were however not observed. The contractile responses to PAF were regained after washing out of the inhibitor. This indicated that as leukotrienes had been shown to be not responsible for PAF receptor desensitization, desensitization was the result of something occurring after PLA<sub>2</sub> activation, but before lipoxygenase activation. Desensitization could therefore be the result of either; a) AA release, or b) as a direct effect of the activation of PLA<sub>2</sub>, leading to its activation being less effective.

Work by Carmo *et al* (1985) on human platelets showed that the direct addition of AA did not prevent the release of eicosanoids and hence tissue response, which suggests that the release of AA does not induce desensitization.

Consequently it appears that desensitization does involve PLA<sub>2</sub> activation, as the inclusion of a PLA<sub>2</sub> inhibitor prevented desensitization. This would support the work of Kamata *et al* (1996) suggesting either down-regulation of the receptor or receptor/G-protein/effector uncoupling after PLA<sub>2</sub> activation as being responsible for tissues desensitization to the effects of PAF (see chapter 1.19).

## **Chapter 5**

### **5.1 Discussion**

The aims of this study were to investigate the actions of PAF *per se* and on PAF's effects on airway responsiveness to ACh and 5-HT. It was hoped that results from previous studies showing hyperresponsiveness to these agents in rat isolated trachea (Warhurst & Court, 1996) could be repeated. A comparison could then have been made between the effects of PAF on the same responses in rat isolated lung tissue. This would allow a comparison of the possible effects of PAF in the larger airways with the possible effects of PAF in the smaller airways. Unfortunately hyperresponsiveness was not demonstrated in either tracheal or lung tissue.

Reasons for this conflict with previous findings were then considered. The same experimental protocols were followed as those by Warhurst and Court (1996), so a slight difference in protocol could not have been responsible. It was noted however that the method of killing the animals sometimes resulted in blood entering into the chest cavity and then into the lungs. As previous studies had shown that PAF can act via blood cells such as eosinophils to cause bronchoconstriction (Clifton *et al.*, 2000) the varying amounts of blood entering the lungs each time a protocol was followed had to be considered as perhaps causing variability in the results. For this reason, an IF technique was developed, from a technique already being used in the study to investigate PAF receptor localization, to show if eosinophils and so probably other blood cells were present within the lung tissue. This IF technique was successful in showing the presence of eosinophils and further modification of the IF technique enabled the identification of

PAF receptors on these eosinophils. This gave at least some explanation into the variability of lung tissue responses, although the variability of tracheal spiral preparations remains unexplained. Another reason for the variability of lung strip responses could be the actions of other smooth muscle fibres which make up the lungs such as vascular smooth muscle, being effected differently by PAF. The only difference between this and previous investigations could have been the source of purchase of the animals, meaning that the animals represented a different gene pool.

Investigation into the bronchoconstrictor properties of PAF showed that PAF induced a homologous desensitization of its receptors to PAF. The use of inhibitors showed that bronchoconstriction induced by PAF is indeed mediated via leukotrienes supporting previous work on rat isolated lung tissue (Voelkel *et al.*, 1985). Investigation into PAF receptor desensitization using inhibitors showed that leukotrienes are not however involved in PAF receptor desensitization. Inhibitors indicated that desensitization was not mediated by PKC activation as suggested by Kamata *et al* (1993), but by something occurring after activation of phospholipase A<sub>2</sub> but before the production of leukotrienes.

IF investigation disproved theories of PAF receptor internalisation being responsible for desensitization to PAF (Kloprogge & Akkerman, 1984). This left the possibilities of either AA or eicosanoids other than leukotrienes being responsible for receptor desensitization or something occurring at the receptor after PLA<sub>2</sub> activation. Previous studies have shown that direct addition of AA does not induce desensitization (Carmo *et al.*, 1985). This indicates that AA release is not responsible for desensitization.

Nakamura *et al* (1991) suggested either down regulation of the receptor or receptor/G-protein/effector uncoupling after PLA<sub>2</sub> activation as being responsible for desensitization. The findings of this study indicate this as being a possible explanation for the homologous desensitization of PAF receptors to PAF after stimulation.

## 5.2 Future studies

Future improvements to this study could involve experimenting with higher concentrations of sphingosine to ensure that protein kinase C was properly inhibited. It may also be useful to use another agent which causes a contraction of lung tissue via leukotrienes such as endothelin 3 (Richter and Sirois, 2000). If the endothelin response could be re-obtained following the washing procedure, it would indicate that the inhibitor was being removed. This would confirm that the effects of lipoxxygenase do not influence desensitization. Another way to determine whether leukotrienes were inducing desensitization could be to introduce leukotrienes to tissue preparations before the addition of PAF and see if a response to PAF is observed.

As well as slight changes in the treatment of the tissues, future studies could also try to eliminate other problems which were encountered in this study. For example the rat lungs could be perfused which would eliminate the possibility of blood cells affecting the results. As well as these improvements, experiments could be carried out using human lung tissue in order to provide results which could be used as a closer comparison to the effects of PAF in asthma.

## Materials

NaCl	
KCl	
K <sub>2</sub> HPO <sub>4</sub>	
MgSO <sub>4</sub> ·6H <sub>2</sub> O	All from Merck eurolab laboratory supplies;
NaHCO <sub>3</sub>	Merck Ltd,
Glucose	Hunter Boulevard,
Sucrose	Magna Park,
CaCl <sub>2</sub>	Leics. LE17 4XN.
Albumin Bovine Fatty acid free	Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset. BH12 4QH.
Acetylcholine Chloride	Sigma-Aldrich Company Ltd.
5-Hydroxytryptamine	Sigma-Aldrich Company Ltd.
L-Ascorbic Acid	Merck eurolab laboratory supplies.
1- <i>O</i> -Alkyl-2-acetyl- <i>sn</i> -glycero-3-phosphocholine (platelet activating factor) Delivered in a chloroform solution which is blown off before PAF is dissolved in GBB-BSA.	Sigma-Aldrich Company Ltd.
D-erythro-Sphingosine, Free Base, Bovine Brain	Calbiochem Biochemicals. CN Biosciences UK, Boulevard Industrial Park, Padge Road, Beeston, Nottingham. NG9 2JR.
{3-[1( <i>P</i> -Chlorobenzyl)-5-(isopropyl)-3- <i>t</i> -butylthioindol-2-yl]-2,2-dimethylpropanoic acid, Na}	
or MK886	Calbiochem Biochemicals.
Arachidonyltrifluoromethyl ketone	or AACOCF <sub>3</sub> Calbiochem Biochemicals.
Sphingosine AACOCF <sub>3</sub> and MK886 are diluted in ethanol before appropriate concentrations are made up for use.	

Acetone	Merck eurolab laboratory supplies.
Normal Rabbit Serum	Sigma-Aldrich Company Ltd.
PAF-R (M-16): sc-8744 Anti-PAF	Santa-Cruz Biotechnology. Autogen Bioclear UK Ltd. Holly Ditch Farm, Mile Elm, Calne, Wiltshire. SN11 0PY
CD154 Anti-human-CD40 ligand	DAKO Ltd. Denmark House, Angel Drove, Ely, Cambridge. CB7 4ET
Normal Goat Serum	Sigma-Aldrich Company Ltd.
Anti-goat IgG with FITC conjugate	Sigma-Aldrich Company Ltd.
Anti-mouse IgG with CY3 conjugate	Sigma-Aldrich Company Ltd.



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